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CALCIUM DISTRIBUTION AND EXCHANGE IN RAT UTERUS

by

Cornelis van Breemen

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled Calcium Distribution and Exchange in Rat Uterus submitted by Cornelis van Breemen in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

Date September 1965

ABSTRACT

The calcium content and distribution of the rat uterus were determined employing flame photometry and Ca^{45} determinations. The total uterine Ca concentration was found to be 2.25 mM/Kg. wet wt., 0.45 mM/Kg wet wt. of which was inexchangeable. The exchangeable Ca could be divided into 0.8 mM/Kg wet wt. extracellular and 1.0 mM/Kg wet wt. intracellular. The concentration of ionic calcium in rat plasma was estimated by equilibrium dialysis as 1.5 mMolar or 53% of the total plasma calcium.

Calculations based on the Nernst equation showed that the intracellular calcium concentration was far below the value it would have if it were ionized and in electrochemical equilibrium with the extracellular calcium. Since Ca^{45} fluxes and net Ca fluxes across the cell membrane could be demonstrated the membrane was assumed permeable to Ca so that in order to explain the low intracellular Ca concentration it was necessary to postulate an active Ca transport.

The metabolic inhibitor I.A.A. and D.N.P. caused a net Ca uptake. It was also shown that I.A.A. did not affect the Ca^{45} efflux, but did increase the influx. The active Ca transport of the uterus therefore appears to be accomplished by an exclusion mechanism.

Cellular Ca binding was measured under conditions of prolonged metabolic inhibition, which abolished both active transport and the membrane potential. Under such conditions approximate association constants were calculated. The values obtained were compatible with the concept that most of the cellular Ca was bound to proteins, but insuf-

ficient to account for the low level of intracellular Ca believed essential for relaxation. Hence metabolically dependent intracellular Ca binding was postulated.

Net and radioactive Ca fluxes were studied during stimulation of the myometrium with acetylocholine and high K solutions. No increase in Ca influx accompanied the contractions and in the case of high K depolarization a decreased Ca^{45} influx and net Ca efflux could be demonstrated. These experiments also showed that contraction was accompanied by a shift of some Ca from a more rapidly exchanging fraction to a more slowly exchanging fraction.

Finally experiments were done to determine the effects of varying concentrations of divalent ions and Na on Ca^{45} efflux. They showed that the Ca^{45} efflux was slowed by omitting Ca from the bathing solution and that the rate could be restored by Ca and Sr and partially by Ba, but that Mg and Na did not affect the Ca^{45} efflux.

The results were discussed with respect to present theories of the roles of Ca in muscular function and membrane transport.

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INTRODUCTION

The importance of calcium ions in muscle physiology was first demonstrated by Ringer in 1883 (1). He showed that frog ventricles in Ca free saline will cease to beat after twenty minutes and that addition of Ca restores spontaneous contractions.

In recent years a vast amount of literature has established that Ca plays essential roles in all three phases of spontaneous contractions, namely; excitation, excitation contraction linkage and the contractile process itself.

Heilbrunn and Wiercinsky in 1947 (2) were the first to demonstrate by injecting various ions into single muscle fibres that an elevated myoplasmic Ca concentration activates the contractile mechanism. Of the cations normally present in tissues in physiologically significant amounts only Ca caused a shortening when injected inside the fibre. Later Niedergerke (3) repeated this experiment, controlling the amount of Ca ions injected by iontophoresis; under these conditions local reversible contractions could be demonstrated. Recently the injection techniques have been refined so that a measure could be obtained for the lowest Ca concentration able to induce a contraction in living muscle fibres. Podolsky (4) showed that in order to induce a contraction of living myofibrils stripped of sarcolemma and isolated in oil it is sufficient to bring them into contact with droplets containing about 2 x 10^{-5} M Ca ions. Thus the threshold concentration of Ca ions for contraction is less than 2 x 10^{-5} M. The unknown extend of dilution made it impossible to know how much smaller the threshold concentration

was. This difficulty was overcome by injecting Ca buffers of relatively high concentrations adjusted to certain concentrations of free Ca.ions. Thus Portzehl,Caldwell and Rüegg (5) injected Ca-EGTA buffers by means of micropipettes longitudinally inserted into isolated living giant muscle fibres of Maia squinado. Contractions occurred when Ca-EGTA buffers adjusted to a free Ca concentration of 5-10 x 10^{-7} were injected. Owing to the high buffer capacity of the solutions injected, the free Ca concentration inside the fibres differed little from the free Ca concentration of the solution injected, although it was diluted approximately thirty times.

The threshold Ca ion concentration for contraction obtained as described above from living muscle fibres agrees very well with data obtained from isolated myofibrils, actomyosin solutions and glycerinated muscle. Weber, Herz and Reiss (6) state that the range of Ca ion concentrations at which myofibrils relax and at which syneresis and ATPase activity of the actomyosin decrease from a maximal to a minimal value extends from concentrations over 10^{-6}M to 10^{-7}M.

Glycerinated skeletal and smooth muscle, in which the cell membranes have been damaged with the expectation that the myofibrils are in direct contact with external solutions, also have a Ca ion concentration threshold for contraction between 10^{-6}-10^{-7}M (7).

The above evidence makes it abundantly clear that Ca ions are involved in the contractile and relaxation processes through changes in their cytoplasmic concentration.

When this concentration exceeds threshold value, between 10^{-6}-10^{-7}M

myofibrils shorten and when Ca ion concentration falls below the same threshold relaxation ensues. The molecular mechanisms whereby Ca initiates contraction have not yet been discovered although much investigation in this field is being done and several theories have been developed (8,9). These theories are based on the ATPase activity of actomyosin and the fact that Mg ions, Ca ions and ATP are necessary for contraction and thus the repetitive making and breaking of cross-bridges between the actin and myosin filaments. However a critical discussion of the molecular theories of contraction is beyond the scope of this introduction.

Studies on Ca movements in isolated muscle tissues are very important, in elucidating the events leading to fluctuations in myoplasmic ionic Ca concentrations.

In 1952 Sandow (10) postulated that the membrane depolarization during the action potential liberates some substance, probably Ca, from the membrane structure itself into the myoplasm. This Ca would then move into the interior of the fiber by a mechanism other than diffusion, possibly an exchange reaction, in order to activate the entire fiber.

Diffusion as a mechanism for activation of skeletal twitch muscle fibers has been eliminated by Hill (11) who showed that this process is far too slow to explain the rapid development of full activity in a twitch by assuming that it is set up by arrival at any point of some substance diffusing from the surface. Hill (11) suggested that some physical or physiochemical process is propagated into

the fiber interior.

Bennet (12) and Porter and Palade (13) demonstrated by electron microscopy that the elements of the triad grouping of the endoplasmic reticulum of skeletal muscle, which surrounds each myofibril in many muscles, appeared to be connected transversely across the fiber. They were the first to hypothesize that some polarized part of the triads conducted excitation from the action potential at the sarcolemma into the fiber. Huxley and Straub (14) and Huxley and Taylor (15) verified this hypothesis by showing that localized contractions of individual sarcomeres could be elicited in response to highly localized subthreshold depolarizations of the muscle surface membrane overlying the triadic elements of the reticulum. Increasing amounts of depolarization caused contractions in increasing numbers of sarcomeres and the direction of spread of excitation was always in the transverse direction across the fibre. Thus the electrical spread inward appeared not to be by propagated action potential, which is all or none in nature. Recently it has been demonstrated that the central tubules of the triads, which run transversely across the muscle fibers are continuous with the extracellular space. (16,17).

Thus presently it is generally accepted that in fast skeletal and probably cardiac (18,19) muscle, which possess an extensive endoplasmic reticulum, excitation is conducted into the fibre interior along the central tubules of the triads. However in smooth muscle (20, 21) and tonic skeletal muscle fibers (22) in which the endoplasmic reticulum is poorly developed and no triad system has been demonstr-

ated, depolarization probably only takes place at the cell surface membrane.

Since the central triad tubules are connected to the extracellular space they contain essentially extracellular fluid and their walls may be regarded as parts of the cell membrane. Since the maximal distance from the central tubule to any part of the contractile fibrils is less than two microns, diffusion of a substance from the central tubular walls is sufficiently fast to explain the rate of activation during a twitch. However there are still several ways in which the cytoplasmic concentration of the activating substance, now accepted to be Ca ions, can be raised above threshold level: 1. increased Ca influx through the cell membrane (also central triad tubular membrane in skeletal and cardiac muscle); 2. release of membrane bound Ca; 3. release of Ca from the endoplasmic reticulum; 4. a combination of two or more of the first three mechanisms. Currently many investigations are being done to determine which of the four above possibilities applies to various types of muscle.

Support for the possibility that Ca entry from the extracellular space during depolarization activates contraction comes from observations of enhanced Ca exchange across the cell membranes of skeletal muscle (23-25), during depolarization. Cardiac muscle studies also support this possibility (26-29). In both tissues the degree of contraction is closely related to the magnitude of the Ca influx (23-29). However certain differences have been observed between cardiac and skeletal muscle. Winegrad and Shanes (28) observed that in guinea pig atria the extra Ca

entry per impulse is proportional to the external Ca concentration, whereas in skeletal muscle (24) the entry during stimulation remains unchanged when the Ca concentration is increased. In both tissues the resting influx is proportional to the external Ca concentration (24,28). Thus it appears that Ca entry during stimulation is derived from sites already saturated at normal Ca levels in skeletal muscle, whereas these sites appear unsaturated in cardiac muscle, (see Nayler, 30). The difference in dependence of resting influx and Ca influx during stimulation on external Ca concentration, indicates that in skeletal muscle at least Ca entry occurs by different routes in resting and stimulated muscle. (24)

Cardiac muscle also differs from other muscle in that only in the heart has a clear cut Na,Ca competition been demonstrated. Lüttgau and Niedergerke (31) studied the tension developed by the heart with different external concentrations of Ca and Na ions; the tension was directly proportional to the concentration of Ca ions and inversely proportional to the square of the concentration of the Na ions. The changes in contractility due to these ions are not associated with corresponding changes in resting or action potentials. Wilbrandt and Koller (32) had shown in an earlier study that this relationship between Ca and Na ions might be expected were the ions distributed according to a Donnan equilibrium between the bathing solution and the cellular surface layer and if the calcium content of the surface layer controls the contraction of the cells. If this surface contains a high density of fixed negative charges and contains only small amounts of cations other than Na and Ca, then its' content of ionic Ca should be a function of the

external Ca concentration:Na concentration squared ratio. In a more recent article Niedergerke (26) interprets the Ca, Na competition by postulating that these ions compete for the same carrier mediating inward transport through the cell membrane, but only the Ca ions which are transported inwards are able to activate the contractile process.

Frank (33) has shown in a very direct manner that external Ca or a Ca fraction in equilibrium with it plays an essential role in excitation contraction coupling during K depolarization in skeletal muscle. He tested the effect of external Ca concentration on the contractile responses of the frog's extensor longus digitus IV incubated in choline Ringer to high potassium depolarization and found that rapid and complete elimination of the mechanical response is obtained when the muscle is placed in a Ca free solution. The rate of loss of the mechanical response could be accounted for by diffusion of Ca ions out of the extracellular space. This loss of contractility was due to a breakdown of the excitation contraction coupling mechanism only since the absence of extracellular Ca did not interfere with the membrane potential or the high potassium induced depolarization and contracture could still be obtained with caffeine (34). However in Na containing solutions Frank found that the mechanical response persisted for several minutes after the removal of extracellular Ca ions (32), and Lüttgau (35) found that it took 5-30 minutes in Na containing Ca free solutions before single fibers lost their contractile response to high potassium. The fact that contractile responses induced by depolarization are lost more slowly than would be expected if it depended solely on the extracellular

Ca concentration has been found also in heart muscle (36) and in smooth muscle (37,38). The explanation given by almost all workers in this field is that the loss of contractility is due to loss of Ca from a membrane bound Ca fraction and the rate of dissociation of this membrane bound Ca is slower than the rate of diffusion out of the extracellular space. However there appear to be other bound Ca fractions which are able to supply Ca for contraction since contractile responses to drugs such as acetylcholine, adrenalin, and caffeine are washed out much more slowly in Ca free solutions than responses to depolarization alone (34, 37-39). Also Frank showed that after responses to depolarization have been eliminated in Ca free solutions they can be temporarily restored by certain divalent ions. Thus these divalent ions appear to release bound Ca during depolarization.

According to the well documented theory developed mainly by Hasselbach and Weber (see 6,41) the cellular fraction from which Ca is released into the myoplasm of skeletal muscle to initiate contraction is the Ca stored in the endoplasmic reticulum. They and other investigators demonstrated that the active reaccumulation of Ca by the microsomal vesicles derived from the endoplasmic reticulum is able to bring about muscular relaxation (6,41). Recently Winegrad (42,43) has obtained some rather direct evidence supporting the above role for the endoplasmic reticulum. He made autoradiographic studies of the localization of exchangeable Ca during contraction and relaxation. Most of the radiocalcium was localised to the center 0.2 to 0.3μ of the I band and part of the A band where the thick and the thin filaments overlap. The relative concentration

of radiocalcium in the two regions could be related to the amount of tension that the muscle was exerting at the time of histological fixation. The greater the tension the greater was the localization to the A band and the smaller the localization to the I band. A study of transverse Ca^{45} distribution (43) showed that most of the A band Ca^{45} is in the myofibrils and most of the I band Ca^{45} between the myofibrils. Winegrad interpreted these data to mean that the I band exchangeable Ca is bound to or associated with the endoplasmic reticulum and the A band exchangeable Ca bound to the contractile filaments. Thus these data support the theory that contraction is initiated by movement of Ca from the lateral cysternae of the endoplasmic reticulum to the myofibrils and relaxation is the result of reaccumulation of Ca by the endoplasmic reticulum.

The data of Winegrad and Shanes (28) and Bianchi and Shanes (23), show that both in cardiac and skeletal muscle, the ratio of the number of Ca ions entering the cells during contraction to the number of myosin molecules is very small. This ratio is in the order of 1:500 for heart and less than that during twitches in skeletal muscle. Since actomyosin is fully activated when Ca is added in a ratio of approximately one Ca ion per one myosin molecule (44) (see also discussions by Davies,8, and Hasselbach,41), the increase in myoplasmic Ca ions which are extracellular in origin is not sufficient to fully activate the contractile system. Frank (34) suggests that care has to be taken in applying data from isolated systems to living muscle since he obtained evidence that the contractile mechanism may only be partly activated during a

twitch so that far less Ca is necessary to initiate contractions.

An interesting possible role for the relatively small extra Ca influx during stimulation is suggested by the experimental results of Frank (45), and Weber, Hertz and Reiss (6). Frank showed that during depolarization a number of divalent cations were able to release Ca from cellular binding sites which he suggested might be located in the endoplasmic reticulum. In accordance with this Weber, Hertz and Reiss demonstrated that the Ca efflux from the microsomal vesicles, derived from the endoplasmic reticulum, is increased at least 15 fold if the external concentration of the divalent cation Ca (this would be cytoplasmic concentration in intact muscle) was raised from 2×10^{-8}M to 10^{-6}M. Thus it seems possible that the influx of small amounts of Ca during excitation could cause the release of a much greater amount of Ca from the endoplasmic reticulum.

Birks (17) suggests a different mechanism for the release of Ca from the endoplasmic reticulum. He suggests that the lumen of the central tubules is connected to the lateral triad sacs, so that the membranes of these sacs are polarized and their depolarization would initiate Ca release.

In smooth muscle the great technical difficulty of isolating endoplasmic membranes is responsible for a complete absence of information of their possible role in regulating muscular activity. Because of the low level of development of the endoplasmic reticulum the bound Ca source for activating the contractile process is presently thought of as cell membrane bound (see reviews by Daniel,46 and 47; Bohr,48; Schild,49; and

Schatzmann,50). However in some cases a well developed endoplasmic reticulum and even tubules running from the surface into the interior of the smooth muscle cell have been observed (21) so that the possibility exists that endoplasmic reticular Ca binding and release is important in regulating smooth muscle activity. On the other hand evidence has been presented, for intestinal (51-55) and vascular smooth muscle (56), that a Ca influx is associated with the initiation of contraction. However as discussed for skeletal and cardiac muscle such evidence does not prove that the source of myoplasmic Ca necessary for activating the contractile processes is exclusively extracellular.

In smooth muscle two different cellular Ca fractions have been shown to be involved with muscle activity. Daniel and Irwin (57) demonstrated that the removal of surface Ca by Ca chelating agents such as EDTA caused the release of less superficial or sequestered Ca which then initiated a contraction of the uterine myometrium. Edman and Schild (37) and Hinke (38) showed that the surface bound Ca is probably involved in mediating depolarization induced contractions and drug induced contractions (acetylcholine and adrenaline) are initiated through release of the deeper or sequestered Ca fraction, since the first response is washed out much more readily than the second (for more complete discussion see Daniel, 47).

The surface bound Ca also has a function in regulating excitability of the cell membrane. Since the frequency and intensity of excitability cycles and the ability of uterine smooth muscle to conduct action potentials regulates myometrial function, surface binding of Ca may be of special importance in this tissue.

The main function of the myometrium is the expulsion of the fetus at the end of pregnancy. The cyclic development of the myometrium is adapted to the performance of this role, with a great increase in weight and actonyosin concentration during pregnancy (59). The development and spontaneous activity of the myometrium are under control of the ovarian and placental hormones, estrogen and progesterone. The influence of these hormones on the uterus is best understood for the rabbit and rat and only these species will be discussed here.

Estrogen secreted by the Graafian follicle and placenta is mainly responsible for increased vascularization growth and actomyosin synthesis, whereas progesterone secreted by the corpus luteum and placenta functions in decreasing spontaneous activity and localizing it, (59). Schofield (59) demonstrated by physiological tests that in the rabbit at the time of mating estrogen is the dominant hormone. Twenty hours later progesterone becomes dominant with respect to its influence of spontaneous activity and remains so until one day before parturition. Csapo (60) demonstrated the importance of the progesterone dominance; it prevents the stimulating action of oxytocin liberated by the posterior pituitary, and also blocks the spread of action potentials thus localizing any spontaneous activity.

The exact cause for the disappearance of the progesterone dominance at the end of pregnancy is not known and the fall in progesterone levels has never been conclusively demonstrated. According to the progesterone theory the disappearance of progesterone results in vigorous contractile responses to endogenous oxytocin.

Although in other species such as the human and the ewe the hormonal regulation of myometrial activity appears more complicated, Schofield (59) believes that in principle the regulating functions of estrogen and progesterone are the same. Although the hormonal regulation of myometrial excitability is still a highly controversial subject it is of special interest that recent experimental results suggest the involvement of Ca in this process. In 1956 Csapo showed that the loss of contractility in Ca free medium is much more rapid for estrogen dominated than for progesterone dominated uteri (61). Recently the work of Kuriyama (62), Casteels and Kuriyama (63), and Marshall (64) has shown that in the rat uterus progesterone dominance and increased external Ca concentrations have the same effects, namely; increased membrane potential, higher threshold of excitation, higher spike amplitude, higher rate of rise and shorter refractory period. Since Casteels and Kuriyama demonstrated that the rise in membrane potential and action potential during progesterone treatment and pregnancy were not due to changes in ionic content, it is probable that progesterone dominance affects the membrane by increasing its affinity for calcium.

As stated before studies of calcium content and movements, employing radio calcium and analytical methods, are very powerful in providing more direct information about the roles of Ca in excitation, contraction and the coupling of these two processes. Since these studies are unfortunately absent for the myometrium, it is the object of this thesis to provide answers to the most basic questions regarding Ca distribution and exchange in the rat uterus.

More specifically answers were sought to the following questions:

1. What is the total Ca concentration?
2. How much of the total Ca is extracellular and how much intracellular?
3. Is the cell membrane permeable to Ca ?
4. Does active Ca transport exist?
5. What is the physical state of the intracellular Ca?
6. Can the existance of specific Ca fractions be demonstrated which function in regulating membrane excitability, contraction and excitation contraction coupling?

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TOTAL UTERINE CALCIUM CONCENTRATION AND CALCIUM EXCHANGE

METHODS

Experimental animals and preparation of tissues.

Female Wistar rats weighing between 50 and 100 grams were pretreated for three days with 100 micro grams diethyl-stilbesterol administered subcutaneously in oil in order to stimulate growth of the myometrium. Each was killed by a blow on the head, its peritoneal cavity opened and the uterine horns dissected free, slit open and placed in K.R. This procedure lasted 2 minutes.

For some experiments the serosa and longitudinal smooth muscle were dissected from the circular smooth muscle and mucosa. The uterine horns as prepared above were placed on filter paper moistened with K.R. with the mucosal side upward. At one end the two layers to be dissected apart were separated by scraping with a piece of razor blade held in a hemostat whereupon the mucosa and circular smooth muscle layer were separated with forceps and pulledaway from the longitudinal smooth muscle layer. This dissection took from 5 to 8 minutes and the ease with which the two layers came apart varied greatly. During the dissection the tissue was continually moistened with K.R.

Solutions.

Krebs Ringer Bicarbonate Solution (K.R.):NaCl 115mM, KCl 4.63mM, $CaCl_2$ 1.5mM, $MgSO_4$ 1mM, $NaHCO_3$ 21.9mM, NaH_2PO_4 1.16mM, Glucose 50mM, pH 7.4, bubbled with 95% O_2 and 5% CO_2.

Tris Buffered Solution (T.S.): NaCl 115mM, KCl 4.63mM, $CaCl_2$ varied, $MgSO_4$ 1mM, Tris Hydroxymethyl Aminomethane 50mM, Glucose 50mM, pH 7.4, bubbled with 100% O_2.

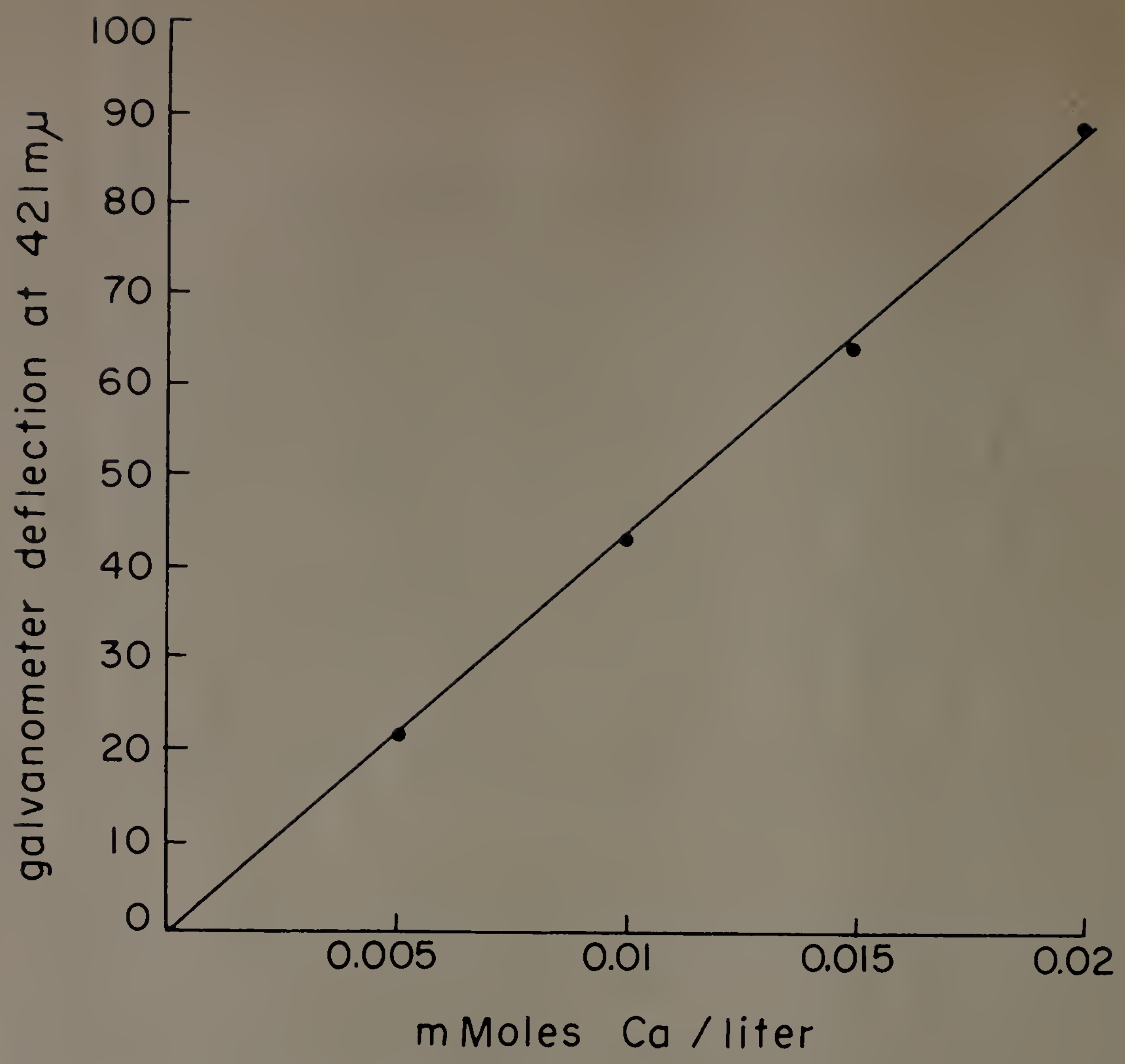


Fig. a. Relationship between galvanometer deflection of Unicam SP-900 and Ca concentration of dilute $CaCl_2$ solutions.

Table a. Recovery data from Unicam SP-900 using internal standard technique.

Ca Concentration of K.R. (mMoles/l)	Recovery (mMoles/l)	% Recovery
0.01	0.01	100
0.01	0.0095	95
1.50	1.43	95
3.00	3.10	103
3.00	3.26	109
5.00	5.20	104
10.00	10.00	100

Mean(± SE) = 100.9% ±1.92%

Drugs.

Ethylene diamine tetra acetic acid Na_3 form, (EDTA).

Determination of total Ca concentration of rat serum and solutions.

Fresh rat serum was diluted one hundred times with deionized water. The calcium concentration of the diluted sample was then determined by the internal standard technique using a S.P. 900 flame spectrophotometer as follows. Galvanometer deflections were obtained in duplicate determinations for the unknown at a wavelength of 421 mμ (D1), and for a mixture of 10 cc unknown and 1cc of known standard at the same wavelength (D2). The standard Ca solutions were prepared by dissolving $CaCO_3$ in 0.1 N HCl to a concentration (c) such that the concentration of the mixture was approximately twice that of the unknown. The deflection due to non-specific interference (Dm) was obtained by obtaining a galvanometer reading for the unknown at a wavelength of 426 mμ. The concentration of the unknown (x) was then calculated from the equation:

$$x = \frac{(D1 - Dm)\ \dot{x}\ C}{11.D2 - 10.D1 - Dm.}$$

This equation follows from a direct proportionality between Ca concentration and galvanometer deflection at 421 mμ over the range of concentrations used and when the interference from other ions remains constant. Such a relationship is demonstrated for solutions of $CaCl_2$ in water in figure a. Recovery data for the S.P. 900 using the internal standard technique as described above are given in table a.

Thus the deflection due to Ca concentration of the unknown is $D1 - Dm = K.x$. The concentration of the mixture of 10 cc of standard and 1cc unknown is $(x + c/10)\frac{10}{11}$ (1). Since the concentration of the other ions is only slightly altered the same proportionality between Ca conc-

entration and galvanometer deflection due to Ca at 421 mμ will hold

thus $D2 - Dm = K . (x + c/10) 10/11$ (2)

Dividing (1) by (2):

$$\frac{D1 - Dm}{D2 - Dm} = \frac{11x}{10(x + c/10)}$$

crossmultiplying:

$$(D1 - Dm)(10x + c) = (D2 - Dm)11x$$

$$-10x(D1 - Dm) - (D1 - Dm)c + 11x(D2 - Dm) = 0$$

$$x(11D2 - 11Dm - 10D1 + 10Dm) = (D1 - Dm)c$$

and $x = \frac{(D1 - Dm)c}{11D2 - 10D1 - Dm}$

Calcium concentrations of experimental solutions were determined using the same method on appropriate dilutions of the solutions in 0.1 N HCl.

Determinations of total tissue Ca concentrations.

Tissues were ashed overnight at 580 - 600 degrees C. and the ash dissolved in 10cc of 0.1 N HCl. A check was made on a known amount of radioactive solution, which showed that no radioactivity was lost during ashing. Five cc of this solution was used to obtain D1 and Dm, and a mixture of the remaining 5 cc and 0.5 cc of standard to obtain D2.

Sample Calculation:

Given: uterus weighing 38mg (wet)
concentration of Ca standard (c) = 0.1mMolar
The uterus was ashed and the ash dissolved into 10 cc of 0.1 N HCl (unknown).
The following galvanometer readings were obtained:
unknown at 421 mμ (D1) = 33
unknown at 426 mμ (Dm) = 4
mixture of 0.5cc standard & 5cc unknown (D2) at 421 mμ = 61

Question: What is the uterine Ca concentration expressed as mMoles Ca per Kg wet tissue?

Solution: Concentration of the unknown $x = \frac{(33 - 4) \cdot (0.1)}{11.66 - 330 - 4} = 0.0086$mMolar

Total amount of Ca in 38 mg of tissue = total amount of Ca in 10 cc of unknown = $10^{-2} \cdot 0.0086$mMoles = $8.6 \cdot 10^{-5}$mMoles

Concentration of Ca in the uterus =

10^{6}mg/kg . 1/38 mg .(8.6×10^{-5}) mMoles = 2.26mMoles/Kg wet wt.

Equilibrium Dialysis.

In a lucite chamber 7.3ml of pooled rat serum was separated by a cellulose dialyser membrane from 7.3 cc of T. S. . The dialyser membrane was immobilised between two lucite grids so that the volumes of the two compartments remained constant. Both compartments were stirred by a magnetic stirrer. The Ca concentrations of the T.S. before and after 12 hrs. of dialysis were determined. The serum concentration of dialysable Ca was taken to equal the T.S. Ca concentration which did not change during dialysis.

Calcium 45 influx.

Uterine horns were incubated in Ca^{45} labelled K.R. for specific durations. They then were blotted by lightly pressing between two sheets of filter paper for about 2 seconds, weighed, and ashed. The ash was dissolved in 2 cc deionized water, one cc of which was spread on a planchette and the remaining cc diluted to 10 cc with 0.1 N HCl for total Ca determination. The planchettes were aluminum discs, which were made water wettable by soaking in detergent for several hours followed by running hot water over them for about ten hours. The surfaces of

the discs were darkened by this procedure which also enabled one to spread one cc evenly over the entire surface of the disc. Although the samples did not run over the edges of the discs, an added precaution was to rub silicone grease on the edges immediately before use. The The samples on the planchettes were dried with infrared light and counted in a Nuclear Chicago model D47 gas flow counter using a micra film end window. The reproducibility of the plating procedure was determined as follows: Five separate 1 cc samples from the same radioactive solution were counted giving an average count of 6,250 with a standard deviation of 1.5%. Only 5,000 counts for each sample were counted.

The radioactivity of the tissues was expressed as millimoles (mM) labelled Ca per kg wet tissue weight by dividing the counts per minute of the tissues by the specific activity of the labelled K.R. in cpm/mM Ca and the tissue weights in kgms.

No corrections for self absorption needed to be made since the samples prepared from the dissolved ash and 100 times diluted labelled K.R. were found to be of infinite dilution for counting purposes.

Sample calculation:

Given: Ca concentration of Ca^{45} labelled K.R. is 1.5mMolar.

Radioactivity of the same solution is 325,000 cpm/cc

Rat uterus weighing 41 mg was incubated at 37 degrees C. in the above Ca^{45} labelled K.R. for 2 hours and then ashed. The ash was dissolved in 2 cc of distilled water, one cc of which was plated and counted. This count was 4,540 cpm (corrected for background).

Question: What was the uterine concentration of labelled Ca?

Answer: The labelled K.R. has a specific activity of

325000cpm/cc x 1000cc/1.50mMoles = 2.17×10^8 cpm/mMole

27mg tissue contains 2 x 4540 = 9080 cpm

Thus the labelled Ca concentration of the uterus was

$$2080 \text{ cpm}/\ 2.17 \times 10^8 \ \frac{\text{cpm}}{\text{mMole}} \ .\ 1\ /\ 27 \text{ mg}\ .\ 10^6 \text{ mg/kg} = 1.57 \frac{\text{mM}}{\text{Kg}}$$

wet tissue weight.

Calcium 45 efflux.

Uterine horns were suspended in test tubes containing 4 cc of K.R. as follows. The solution in the test tube was bubbled with carbogen (95% O_2, 5% CO_2) through a narrow glass tube (aerator) open on both ends and held by a rubber stopper, which rested on the top of the test tube. The uterine horn was suspended on a small U shaped stainless steel wire. The ends of this steel wire could be brought together by means of a hemostat and then inserted into the free end of the aerator. This arrangement allowed transfer of the tissue from test tube to test tube as well as rapid transfer from one aerator to another. The uteri were loaded in a test tube containing Ca^{45} labelled K.R. for a time long enough to exchange almost all the exchangeable Ca, which was 2 to 3 hours. The tissues were then rinsed by dipping into two successive tubes containing non-labelled K.R. for a total time of 1 to 2 seconds. They were then transferred to a clean aerator and passed through a series of test tubes containing 4 cc non-labelled K.R.. They remained in each for an accurately measured period of time.* At the end of the efflux period the tissues were blotted, weighed, and ashed. One cc of each efflux tube was dried on a planchette and counted. To avoid the necessity of corrections for self absorption the ash was dissolved in K.R. instead of water, for plating and counting. Also the loading solution was diluted in K.R. to be counted.

*for consideration of backflux see page 30.

Sample calculation of efflux data:

Since efflux experiments usually had 12 collection periods, with times varying from 3 minutes initially to 30 minutes finally, a simplified experiment with imaginary data will be calculated in order to save space while still showing all calculations.

Given:

30 mg rat uterine horn was loaded for 2 hrs. in Ca^{45} labelled K.R. containing 3,000,000 cpm/cc and 1.5 mM Ca / litre at 37 degrees C. It was then passed through 3 efflux tubes (1,2,3) remaining in each for 10 minutes. One cc samples from tubes 1, 2, and 3 contained respectively 8,000 cpm, 5,000 cpm, and 3,000 cpm. The tissue after the 30 minutes of efflux contained 40,000 cpm.

Question:

Calculate:

a) the concentration of radioactivity in the uterus at various times during the efflux

b) the average rates of efflux during each of the collection periods

c) the concentration of labelled Ca before efflux

Answer:

a) Amounts of radioactivity in tubes 1, 2 and 3 (each containing 4 cc) were respectively 32,000 cpm; 20,000 cpm; and 12,000 cpm.

After 30 minutes of efflux, the uterus contained 40,000 cpm
" 20 " " " " " " 40,000 + 12,000 = 52,000 cpm
" 10 " " " " " " 52,000 + 20,000 = 72,000 cpm
" 0 " " " " " " 72,000 + 32,000 = 104,000 cpm

b) The average rate of efflux during the first 10 minutes was 32,000/10 cpm/minute, during the second 10 minutes was 20,000/10 cpm/min., and during the third 10 minutes was 12,000/10 cpm/minute.

c) The specific activity of the Ca^{45} labelled K.R. was 3,000,000 cpm/cc x 1,000cc/1.5mM = 2×10^9 cpm/mM. Thus the labelled Ca concentration of the uterus before efflux was:

$$\frac{104{,}000 \text{ cpm} \times 10^6 \text{ mg/kg} \times 1/30 \text{ mg}}{2 \times 10^9 \text{ cpm/ mM}} = 1.73 \text{ mM/Kg wet weight}$$

Loss of total tissue calcium into Ca free solution.

Uteri were incubated in Ca free T.S. for various times and then analysed for total Ca concentration.

General observations on tissue calcium studies.

All glassware used was Pyrex, which does not contaminate solutions contained in it with Ca, provided it is properly cleaned. All stock solutions were kept in polyethelene bottles under refrigeration.

The cleaning of glassware consisted of rinsing in tap water, soaking in detergent, rinsing in tapwater and distilled water, soaking in 5 to 10% nitric acid made up with distilled water, and finally rinsing in distilled and demineralized water. The distilled water supply was checked and found to be free of Ca contamination. Later during the course of these experiments all glassware used for Ca determinations and for experiments with Ca free solutions were after the above procedure, soaked in Na_4EDTA solution made up with distilled water followed by rinses in distilled water and demineralized water.

Also extra care is needed in handling and dissection of the tissues since the Ca concentration of the tissues is increased with the slightest amount of damage. As will be demonstrated in the results dissection of the longitudinal smooth muscle caused such a large and variable increase of tissue Ca as to make dissection for Ca exchange studies impractible.

All experiments were carried out at 37 degrees C. unless stated differently.

Histological slides of rat uteri.

Haematoxylin Eosin stained sections of uterine horns from estrogen pretreated immature female rats were prepared according to routine techniques (1).

* Backflux during collection periods is not considered to significantly affect the Ca^{45} efflux curves. This may be shown as follows from the experiment illustrated in fig. 6. In this experiment the greatest per cent loss of Ca^{45} occurred during the collection period from the 26th to the 46th minute.

If mixing of Ca^{45} and Ca^{40} occurs, the rate of Ca efflux will be $(\text{cpm/minute})_{\text{out}} \times (\text{mMole/cpm})_{\text{tissue}}$ which at steady state is equal to rate of Ca influx which is $(\text{cpm/minute})_{\text{in}} \times (\text{mMole/cpm})_{\text{medium}}$.

Thus $\dfrac{(\text{cpm/minute})_{\text{in}}}{(\text{cpm/minute})_{\text{out}}} = (\text{mMole/cpm})_{\text{tissue}}/(\text{mMole/cpm})_{\text{medium}}$

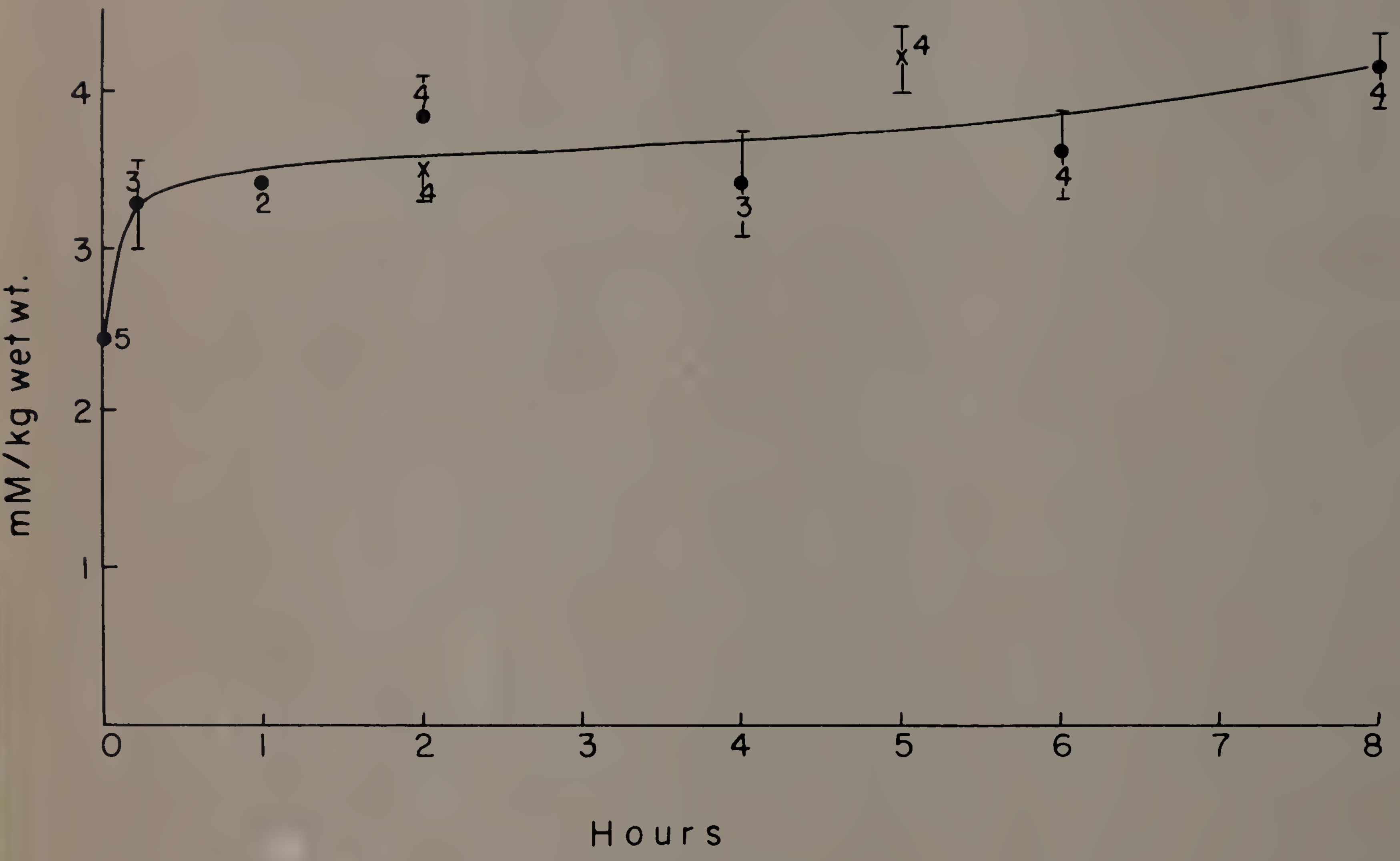
Since the average tissue wt. was 35 mg, the tissue concentration of exchangeable Ca was 1.8mM/kg tissue, at 36 minutes the cpm of the tissue and the 4 cc medium were respectively 15,000cpm and 5,000 cpm, and the Ca concentration of the medium was 1.5 mM/litre, at the middle of the collection period $\dfrac{\text{rate of } Ca^{45} \text{ influx}}{\text{rate of Ca 45 out}} =$

$$1.8 \times 10^{-6} \frac{\text{mM}}{\text{mg}} \times 35 \text{ mg} \times 1/15{,}000\text{cpm} \times 1/4 \text{ ml} \times 1 \text{ ml}/1.5\text{x}10^{-3}\text{mM} \times 5{,}000 \text{ cpm}$$

$= 63/14{,}000$

Thus during this experiment the largest average backflux of radio activity as a % of efflux of radioactivity was estimated to be slightly less than 0.5%, which would still be considerably smaller than the experimental variation.

One experimental finding which supports the assumption of negligible backflux is illustrated in fig.17. In this experiment Ca^{45} could be released from a superficial Ca fraction after it had been exposed to labelled medium. The fact that no Ca^{45} was released on second K depolarization indicates that the Ca^{45} in the efflux medium was too dilute to significantly relabel this Ca fraction.


mM/kg wet wt.
4
3
2
1
0
1
2
3
4
5
6
7
8
Hours
5
3
2
4
4
3
4
4
4

RESULTS

Total Ca concentration of uterine horns when incubated in K.R. containing 2.5 mMolar Ca.

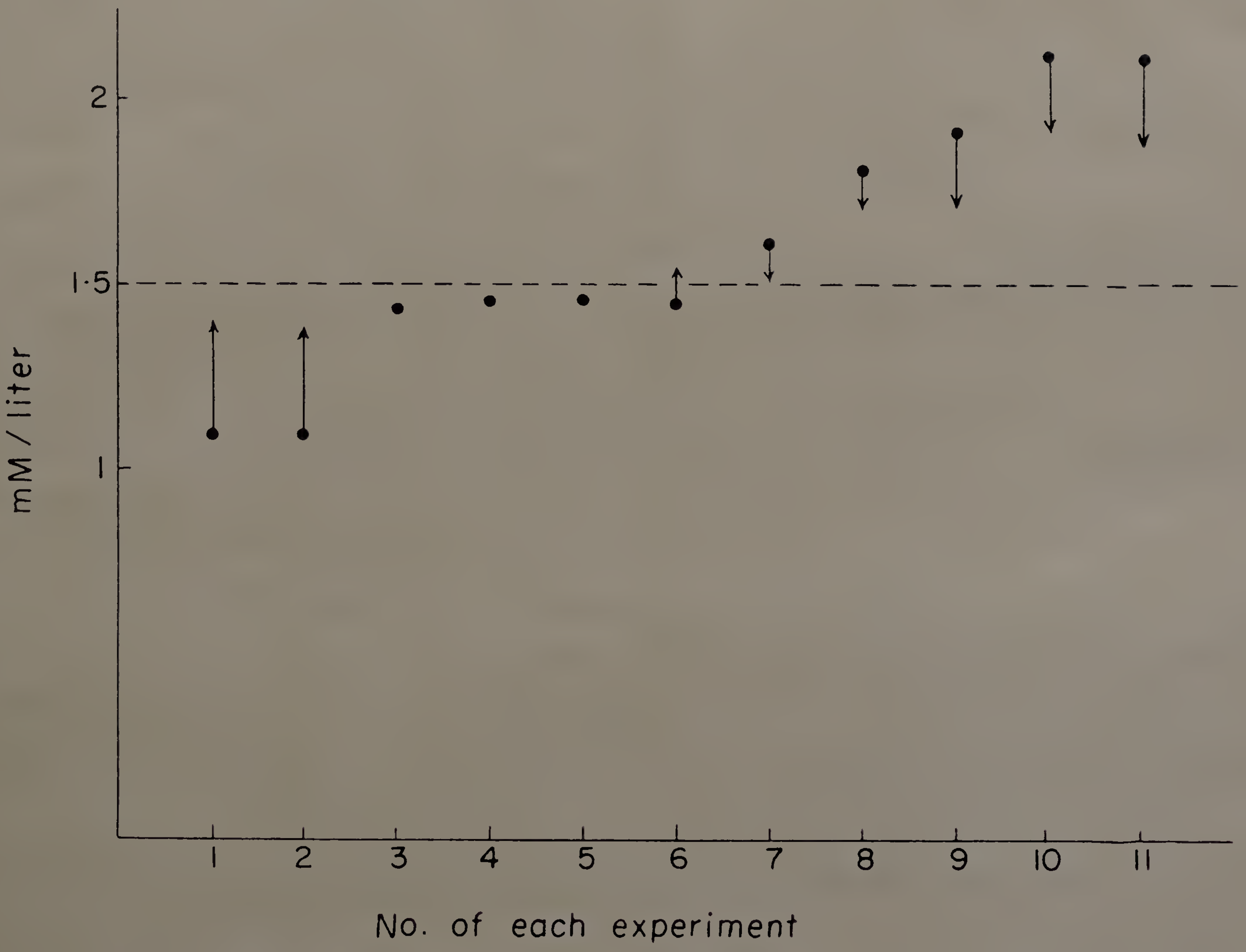
Initial total tissue Ca determinations showed that uteri incubated in the usual Krebs Ringer solution containing 2.5 mMolar contained more Ca than uteri which were analyzed immediately after removal from the rats. Fig. 1 demonstrates this rise in total tissue Ca concentration and also shows that it could not be prevented by omission of phosphate from the medium. Since the uteri were not damaged by dissection, and since 2.5 mMolar Ca is about twice as high as human ultrafiltrable plasma Ca (2), it was suspected that the in vitro rise in tissue Ca was due to too high a Ca concentration in the usual Krebs Ringer solution. This possibility was further investigated.

Total and free serum Ca determinations.

The results of the equilibrium dialysis are given in Fig. 2. Each arrow indicates the shift in Ca concentration of the Tris buffer solution (T.S.) which took place during 12 hours dialysis against pooled rat serum. These results indicate the free serum calcium concentration to be 1.5 mMolar. Since the capillary walls do not constitute a selective electrolyte barrier (3) the free calcium concentration of the extracellular space can be assumed to be approximately 1.5 mMolar also. Thus

Fig. 1. Total Ca concentration of rat uterine horns incubated in K.R. containing 2.5 mMolar Ca (•), and in phosphate free K.R. containing 2.5 mMolar Ca (X). The numbers by each point indicate the number of horns analyzed. Verticle bar extending in one direction from a point indicates one standard error.

Equilibrium dialysis of rat serum with buffered test solution. Arrows indicate the change of calcium concentration in the test solution.



to more closely approximate in vivo conditions in the Ca exchange studies the calcium concentration of the Krebs Ringer bicarbonate was changed from the usual 2.5 mMolar to 1.5 mMolar.

The total serum Ca concentration determined by dilution in double distilled water was 2.7±0.18 (6) and by deproteinization with 10% TCA 2.9±0.13 (5). (All values are given ± standard error with number of determinations in brackets)

Ca gained as a result of dissection.

The longitudinal smooth muscle and some circular smooth muscle were dissected away from the remainder of the uterine horns. The smooth muscle was then either immediately analyzed for total Ca or incubated in K.R. (1.5 mM Ca) before analysis. The curve in Fig. 3 shows that the damage due to dissection was sufficiently great to cause a net uptake of Ca. Four other pieces of isolated uterine smooth muscle were first incubated in K.R. for thirty minutes followed by an hour in Ca^{45} labelled K.R. and were then analyzed for labelled Ca content. As can be seen from Fig. 3 the gained Ca appears to be exchangeable.

Fig. 2. Equilibrium dialysis of pooled rat serum with Tris buffered solution containing varying concentrations of Ca. Each point indicates mMoles of Ca per liter of Tris buffered solution (ordinate) before each experiment. The tips of the arrows indicate the Ca concentrations of the Tris solutions after 12 hours dialysis against pooled rat serum. Absence of arrows means no change in Ca concentrations.

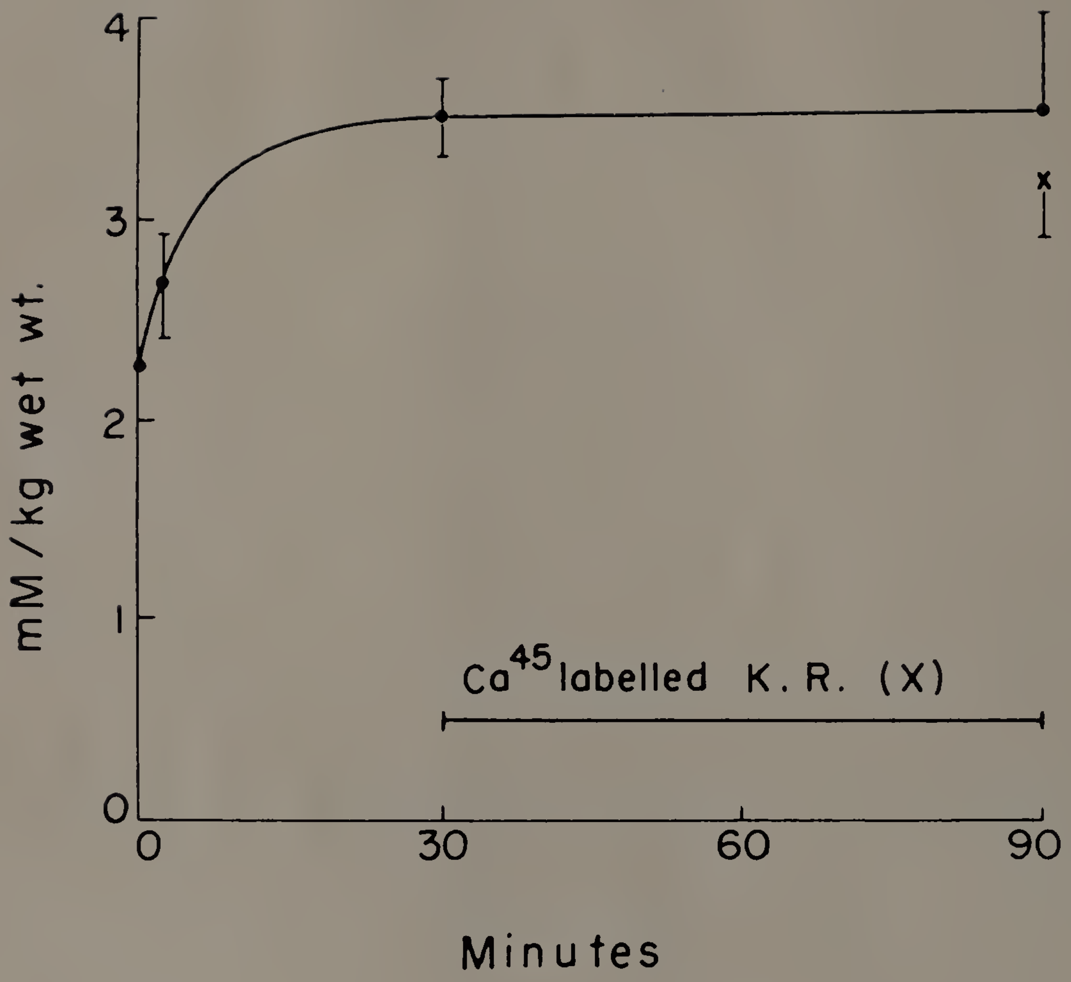
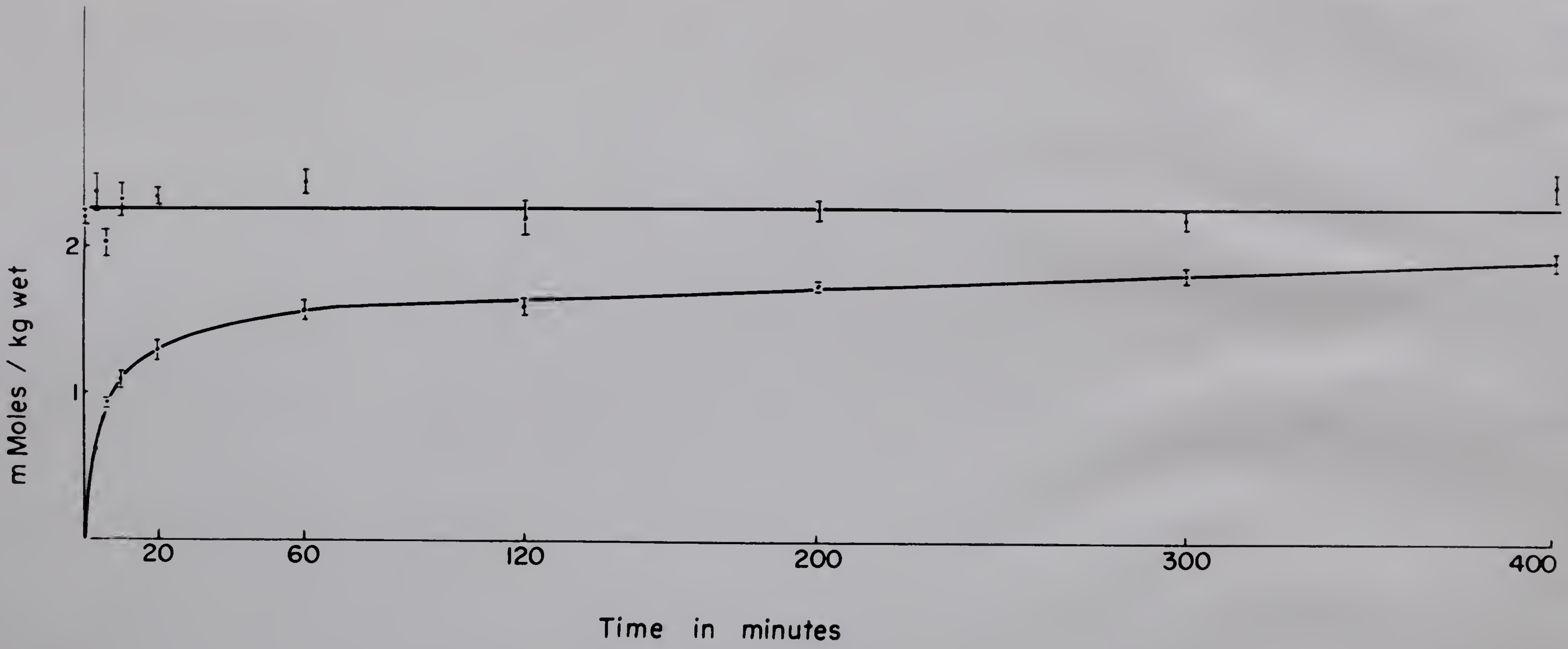

4
3
2
1
0
mM / kg wet wt.
Ca45 labelled K. R. (X)
x
0
30
60
90
Minutes

Fig. 3. Net Ca uptake by dissected uterine smooth muscle (·) from K.R. (1.5 mM Ca). Uptake of labelled Ca during time indicated by the horizontal bar (X). Each point is an average of 4 determinations. Vertical bars indicate twice the standard error, except at 90 minutes when they indicate one standard error.


m Moles / kg wet
2
1
20
60
120
200
300
400
Time in minutes

Examination of histological slides from immature estrogen treated rats indicated that more than 75% of the uteri was smooth muscle.

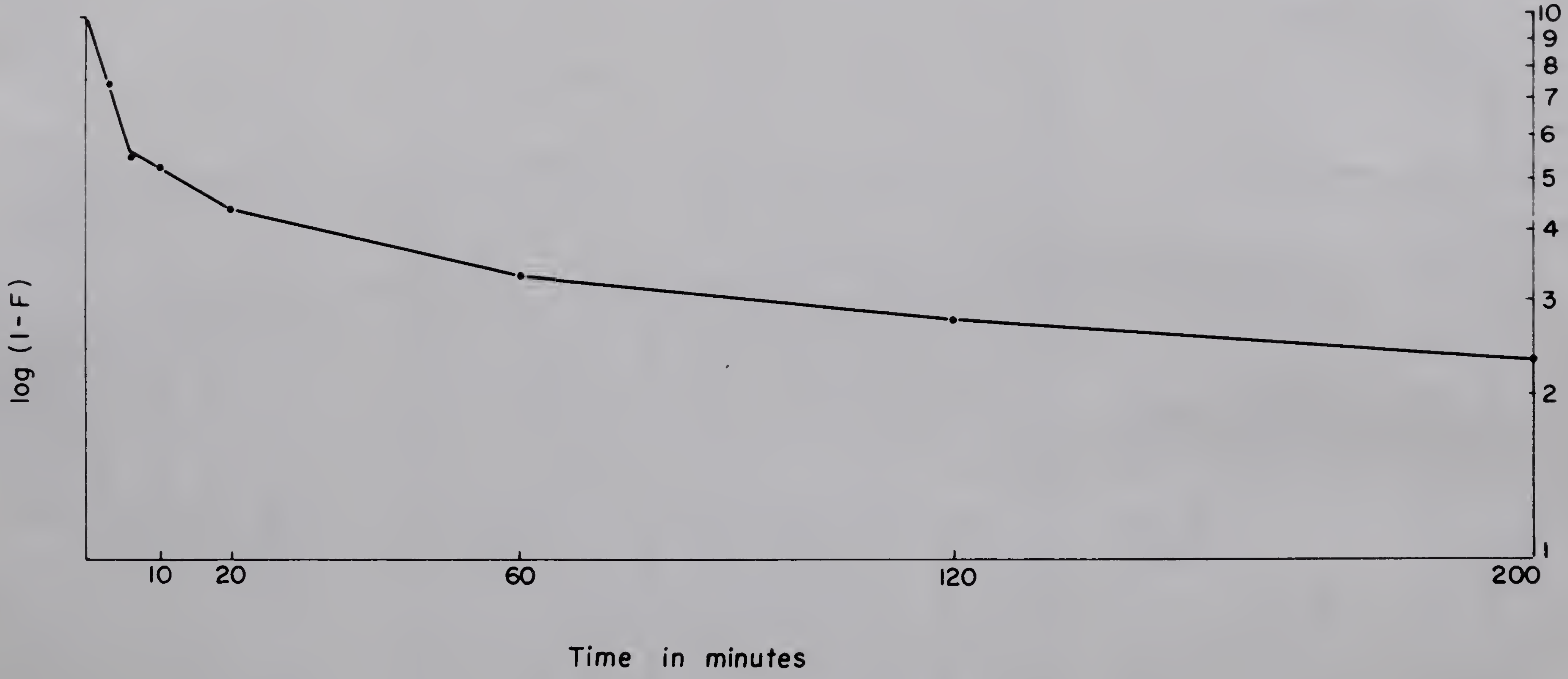
Since results from dissected myometrium were more variable and would in part be attributable to an unphysiological Ca fraction (i.e. gains due to dissection) it was decided to use whole uterine horns in the Ca exchange studies.

The uptake of total and labelled calcium in whole uterine horns.

The change in tissue content of total and labelled Ca with time is given in Fig. 4. At an external Ca concentration of 1.5 mMolar, the in vivo steady state for Ca is not apparently upset as the total calcium concentration remains constant at a value of approximately 2.25 mMols/kg wet tissue weight for many hours. The constancy of the total tissue Ca concentration allows the uptake or loss of radio active Ca to be interpreted as true Ca exchange.

The uptake of labelled Ca as shown in the lower curve of Fig. 4 occurs at an initial rapid rate, which is followed by a continual slowing of the uptake rate. After 2 hours the uptake of labelled Ca is so slow, that it can not be differentiated from a possible small net

Fig. 4. Upper line shows the total Ca concentration of rat uterine horns incubated, for times as indicated, in K.R. (1.5 mM Ca). The lower curve shows the time course of Ca^{45} uptake expressed as mMoles labelled Ca per kg wet tissue. Each point is the average obtained from 5 or 6 tissues. The radioactive and total Ca values were obtained from the same uteri.

log (1 - F)
10
9
8
7
6
5
4
3
2
1
10
20
60
120
200
Time in minutes

gain. At this time between 0.4 to 0.5 mMols Ca/kg wet wt. has not exchanged. Thus about 0.45 mMols/kg wet wt. is inexchangeable or very slowly exchangeable Ca.

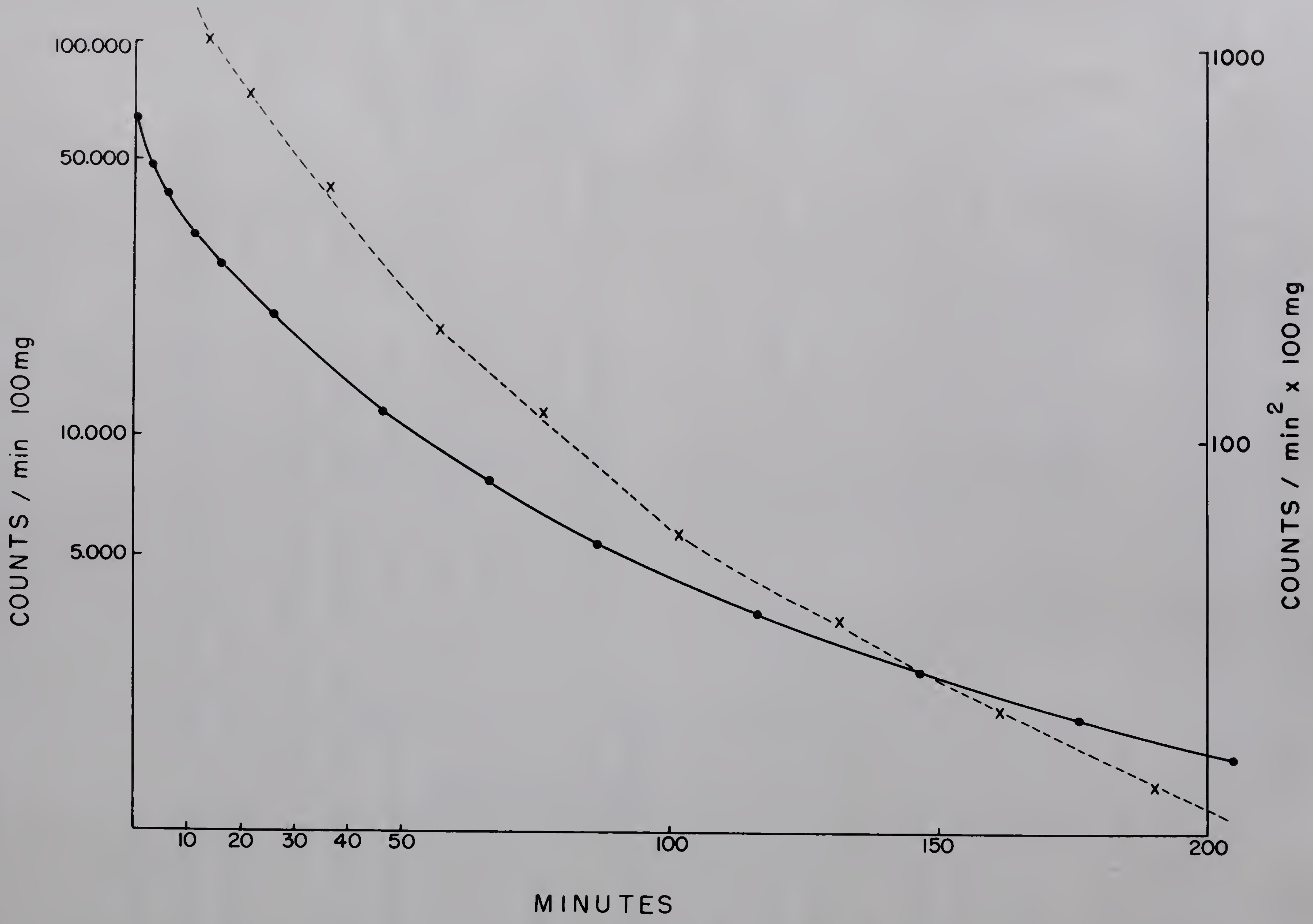
Fig. 5 shows the uptake of labelled Ca plotted as the logarithm of the unexchanged fraction vs. time. It is obvious that the uptake of labelled Ca does not follow first order kinetics, in which case such a plot would yield a straight line over the entire range of data. Also it can not easily be treated as a sum of a few exponential processes since to justify such an attempt at least the latter part of the curve should be a straight line. The many possible causes for the observed kinetics will be discussed later.

Efflux of labelled Ca.

The loss of Ca^{45} from uteri incubated for three hours in Ca^{45} labelled K.R. is illustrated in Fig. 6. The solid curve shows the log of the radioactivity in the tissue vs time. The dashed line represents the rate of Ca^{45} loss at any time. The plots are not linear and do not become parallel at any time, indicating that the Ca was not emerging from a single, well mixed compartment with first order kinetics.

The results obtained from Ca influx and efflux show that rat uterine exchange kinetics can not be fitted to a unique sum of first order processes, making it impossible to distinguish tissue Ca fractions on this basis.

Fig. 5. Uptake of Ca^{45} by rat uterine horns plotted as $\log_{10}$ (1 - labelled Ca concentration / total Ca concentration). The points shown were calculated from the values given in Fig. 4.


100.000
50.000
10.000
5.000
COUNTS / min 100 mg
1000
100
COUNTS / min² x 100 mg
10
20
30
40
50
100
150
200
MINUTES

Loss of total Ca into Ca free solution.

The time course of the loss of total Ca into Ca free solution is illustrated in Fig. 7. The main points of this experiment are that Ca is lost at an initially rapid rate and that Ca free medium is unable to extract a fraction of the tissue Ca which is approximately equal to the inexchangeable fraction i.e. about 0.5 mM/Kg wet wt. Note that the presence of lmMolar iodoacetic acid does not affect the net Ca loss into Ca free solution.

From the above experiments it can be seen that the initial rapid exchange and loss of Ca involves about 0.9 mM/Kg wet wt. A reasonable assumption is, that this is mainly extracellular Ca and to a small extent cellular Ca. Taking the extracellular space for rat uterus to be 460 ml/Kg wet wt. (4), the minimum ionic extracellular Ca would be 0.69 mM/Kg wet weight. Thus the extracellular Ca would lie between 0.69 and 0.90 mM/Kg wet wt. and was arbitrarily estimated to be 0.8 mM/Kg wet wt., thus allowing for a small amount of extracellularly bound Ca. The only large error in the above estimate could arise if a considerable amount of the more slowly exchangeable Ca were bound in the extracellular space. This possibility was investigated by comparing the loss of tissue Ca during a 10 minute exposure to Ca free K.R. with the loss of tissue Ca during a 6 minute exposure to Ca free K.R. containing

Fig. 6. Ca 45 efflux from rat uteri. Uterine horns were loaded in Ca^{45} labelled K.R. for 3 hours and then passed through a series of inactive K.R. solutions. The solid curve represents the loss of tissue Ca^{45} with time and the dashed curve the rate of Ca^{45} loss vs. time. Each point is the average of 5 determinations.

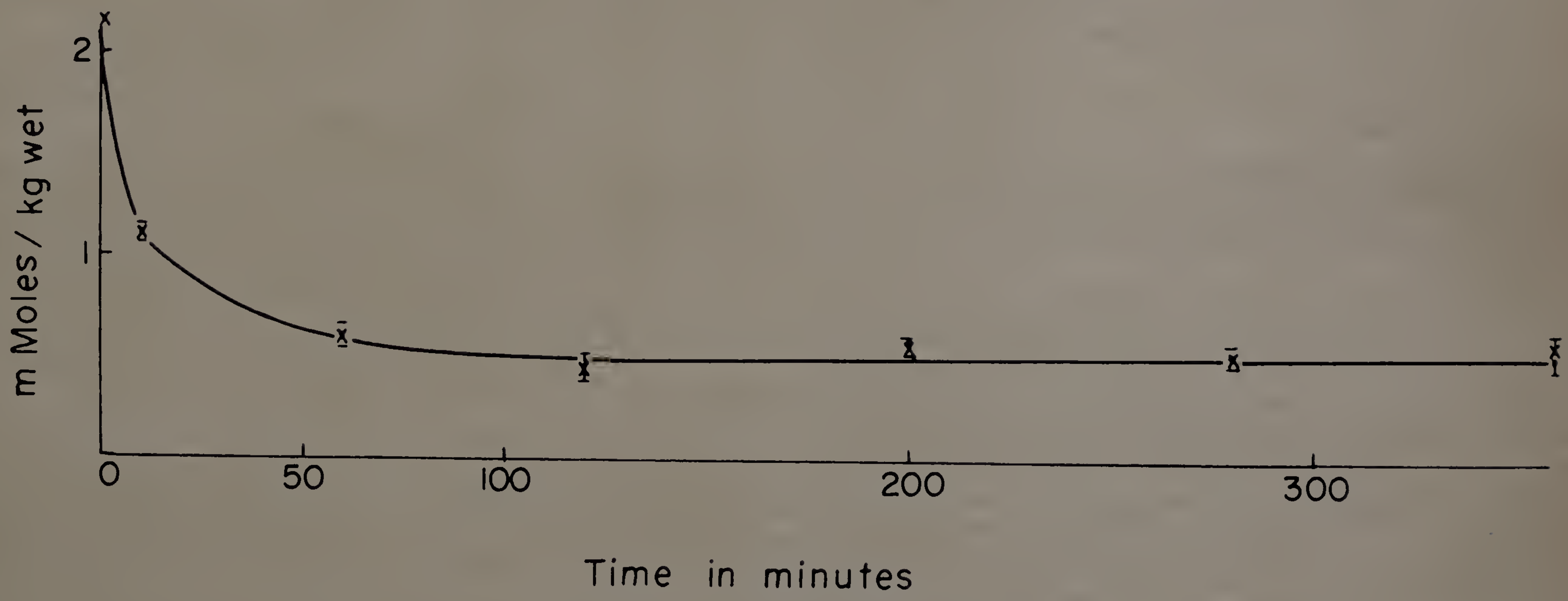

m Moles / kg wet
2
1
0
50
100
200
300
Time in minutes

Fig. 7. Loss of total Ca from rat uterine horns incubated in Ca free solution (T.S.) (·), and Ca free T.S. containing 1 mM IAA (X). Points are averages of 4 determinations for each procedure. Where crosses and dots are superimposed only crosses are drawn in. Vertical bars are twice the S.E.

5 mM Na_3EDTA (i.e. at pH 7.4) followed by 4 minutes in Ca free K.R.. This experiment is based on the assumption that EDTA which has a volume of distribution resembling that of sucrose will remove only superficially bound extracellular Ca, for which some evidence is available (5.6). Also in conjunction with this work it was shown that a 10 minute exposure of serum to Chelex 100 (Bio-Rad), which has chelating properties similar to EDTA, removed at least 93% of the protein bound Ca. However in the above described experiment the loss of tissue Ca was the same, within experimental error, in both cases. The total Ca after Ca free exposure was 1.09 ± 0.04 (4), and Ca free plus EDTA 1.08 ± 0.10 mM/kg wet wt. (4), indicating that a large amount of extracellularly bound Ca is unlikely.*

The Ca exchange and loss studies lead to the following tissue

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* A short experiment was done to demonstrate that EDTA would remove extracellular bound Ca. Rat Achilles tendon and fascia about 6 mg each were incubated in Ca^{45} labelled K.R. for one hour. Tendon and fascia from one side of the animal were then exposed to Ca free K.R. for ten minutes and the same tissues from the other side to Ca free K.R. containing 5 mM EDTA also for ten minutes. The Ca free K.R. had reduced the labelled Ca concentration in the tendon to 0.38 mM/kg wet wt. and in the fascia to 0.54 mM/kg wet wt., whereas the respective values for EDTA containing Ca free K.R. were 0.075 mM/kg wet wt. and 0.161 mM/kg wet wt. Since tendon and fascia are acellular but do contain bound Ca (7) this is considered to be a demonstration that EDTA is able to remove most of any extracellular bound Ca.

Ca distribution: Extracellular Ca 0.8 mM/Kg wet wt., Cellular exchangeable Ca 1.0 mM/Kg wet wt., inexchangeable Ca 0.45 mM/Kg wet wt.

ACTIVE TRANSPORT OF CALCIUM

METHODS

Solutions.

The Na concentration in K.R. or T.S. was raised in some instances to 200 mMolar by adding NaCl. Solutions containing 50 mMolar and 10 mMolar NaCl were prepared by substituting equimolar amounts of choline chloride for NaCl in the 200 mMolar NaCl solution.

In experiments designed to test the effects of metabolic inhibition, 1 mMolar iodoacetic acid (IAA) was added to the incubation solutions (K.R. and T.S.).

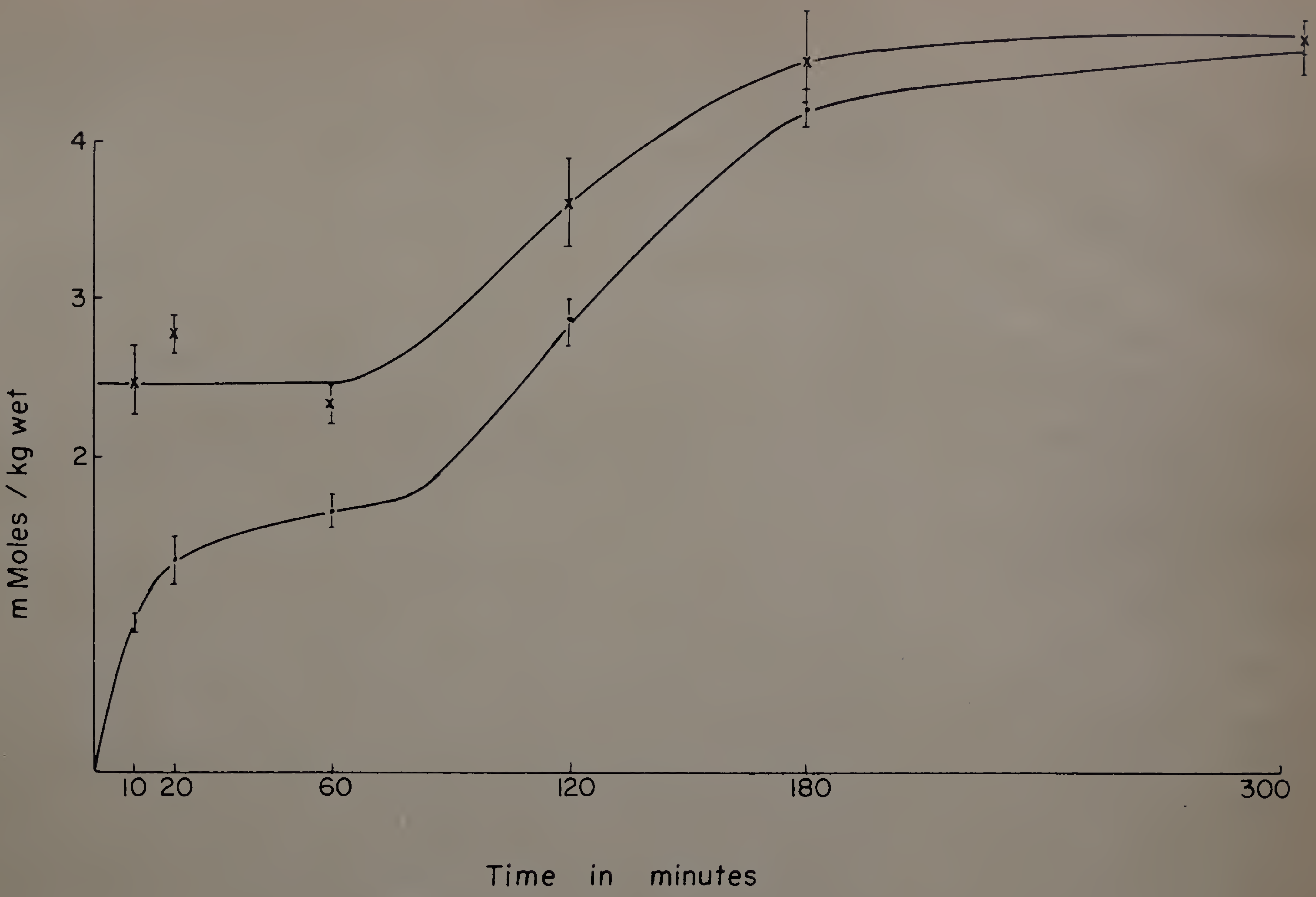
Effects of 10^{-3} M Quabain were also tested.

Method for estimating total Ca from Ca^{45} measurements.

Tissues were incubated in Ca^{45} labelled K.R. or T.S. for 2 hours in order to label the exchangeable Ca before incubation in Ca^{45} labelled experimental and control solutions. Since all the solutions had the same known specific activity, analysis at the end of the experiment yielded values for total exchangeable Ca. To obtain values for total tissue Ca, 0.45 mM/kg wet wt. was added to correct for the inexchangeable fraction. This method (called Ca^{45} method) gives values comparable with the flame spectrophotometer (see table 1), but is more convenient and gives less variation. Where this method is used it will be specifically mentioned; all other total Ca determinations were done as described before.

Tissues and analytical procedures.

These were the same as for chapter one except that for the Na replacement studies the uterine horns were not slit open.


4
3
2
m Moles / kg wet
10
20
60
120
180
300
Time in minutes

Details of the experiments will be given in results.

RESULTS

The effect of IAA on Ca^{45} uptake and the total tissue Ca concentration.

If the internal Ca ions were at electro-chemical equilibrium with the extracellular Ca ions, the Nernst equation, $Ca_i = Ca_o \exp. -\frac{2VF}{RT}$, would predict an intracellular Ca ion concentration of 67 mMolar.

Ca_i - intracellular Ca ion concentration.

Ca_o - extracellular Ca ion concentration.

V - electrical potential difference across the cell membranes, inside minus outside, and for this calculation taken to be - 50 mV.

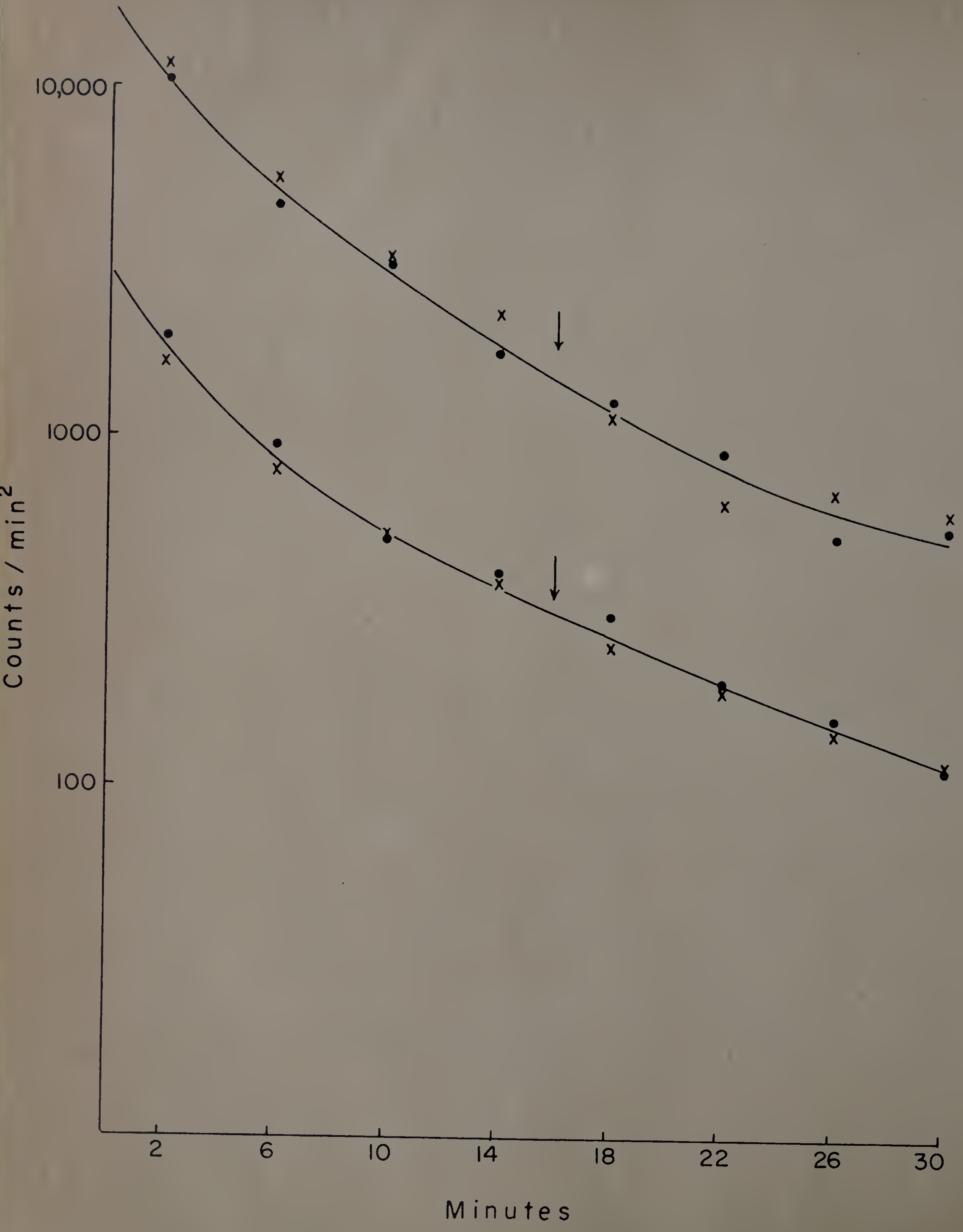
F - 96,496 coulombs/mole.

R - 8.31431 joules/degree C. mole.

The concentrations in this equation are used as approximations of the activities.

Even if all the cellular exchangeable Ca were ionized (1 mM/kg wet wt.), which is known to be incorrect, the intracellular Ca concentration would still only be 2.9 mMolar. It is thus evident that a large electrochemical gradient for Ca exists across the uterine cell membrane. The probability that this gradient depends on the availability of metabolic energy was investigated by studying the effect of IAA on the Ca uptake. Fig. 8 shows the uptake of total and labelled Ca when

Fig. 8. Uptake of labelled (•) and total Ca (X) by rat uteri when incubated in K.R. (1.5 mM Ca) labelled with Ca^{45} and containing 1 mMolar IAA. Radioactive and total Ca concentrations obtained from the same uteri. Each point is an average of 4 determinations. Bars = 2 times S.E.


10,000
1000
100
Counts / min²
2
6
10
14
18
22
26
30
Minutes

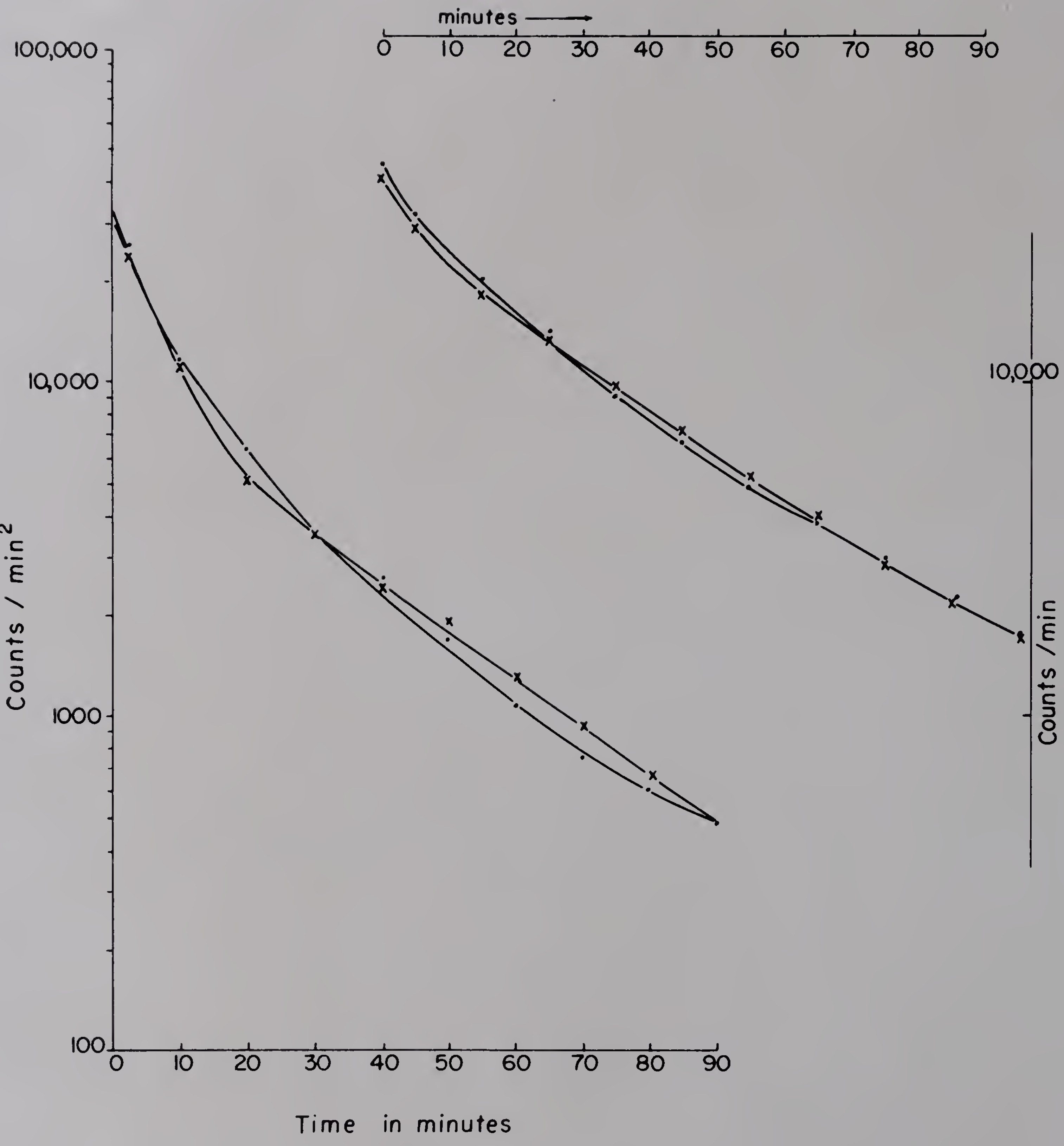
uteri were incubated for various times in Ca^{45} labelled K.R. containing 1.0 mMolar IAA. These curves demonstrate that the action of IAA has a latent period of about 70 minutes before it causes a net uptake of 2 mM Ca/kg wet wt.

To test if the IAA induced Ca uptake could be reversed by reincubating in K.R. some uteri were exposed to IAA for 3 hours followed by K.R. for another 3 hours. Analysis showed these uteri to contain 4.9 mM Ca/kg wet wt. ± 0.13 (4). Uterine horns from the same rats incubated in K.R. containing 1 mM IAA for 3 hours and then analyzed contained 4.47 mM Ca/kg wet wt. ± 0.18 (4). Thus the IAA-induced Ca uptake was not reversed upon reincubation in K.R.

Effect of IAA on Ca^{45} efflux.

When the efflux medium is changed from K.R. to K.R. containing 1 mMolar IAA there is no change in the rate of efflux as can be seen in Fig. 9. However if the rise in total Ca were due to a decreased efflux this would be expected due to the delayed action of IAA. Thus to study the efflux over the period during which the net Ca uptake occurred, the experimental uterine horns were loaded for 80 minutes in Ca^{45} labelled K.R. and for an additional 40 minutes in Ca^{45} labelled

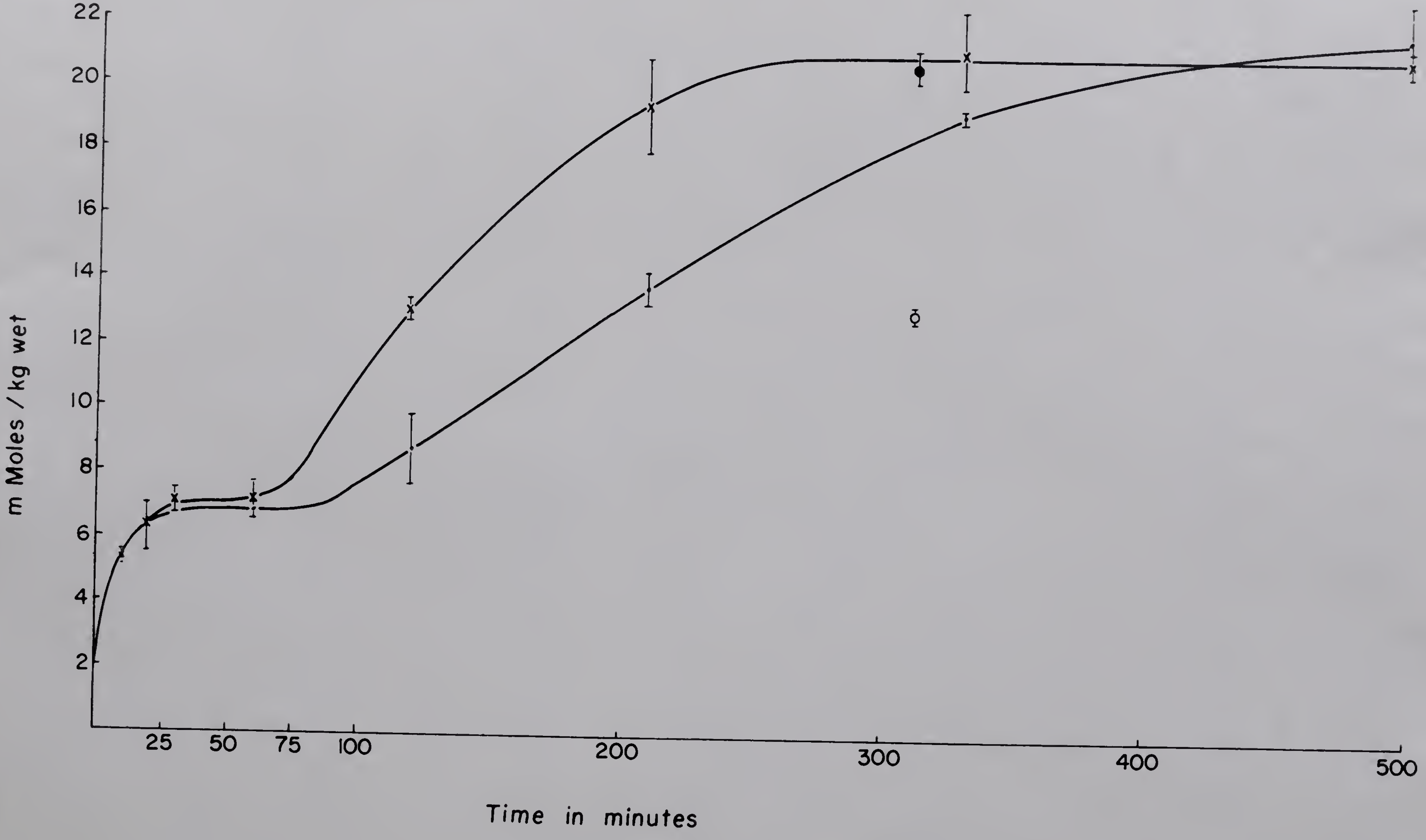
Fig. 9. The effect of 1 mMolar ouabain (upper curve), and of 1 mMolar IAA (lower curve) on the rate of efflux of Ca^{45} from rat uterine horns. The drugs were added at times indicated by arrows to efflux media of experimental horns (X). Controls (•) remained in K.R. during entire efflux period. Points of upper curve are averages of two experiments, and of lower curve of four experiments. Values of upper curve were multiplied by 10 for convenience of presentation.


minutes
0
10
20
30
40
50
60
70
80
90
100,000
10,000
1000
100
Counts / min^2
Time in minutes
10,000
Counts / min

K.R. containing 1 mM IAA. The experimental tissues were then effluxed in inactive K.R. containing 1mM IAA for 95 minutes. These efflux curves were compared with control efflux curves obtained from uterine horns from the same rats and loaded and effluxed for the same times but not exposed to IAA. Fig 10 is a typical experiment of 6 such experiments. The tissue Ca concentration at time 0 was calculated by adding 0.45 mM/Kg wet wt. for the inexchanged Ca to the labelled Ca concentration at that time; the tissue Ca concentration at the end of the 95 minute efflux period was determined by flame photometry. During the efflux period the Ca concentration of the experimental horn rose from 2.1 mM/Kg wet wt. to 4.3 mM/Kg wet wt., whereas the corresponding control values were 2.2 mM/Kg wet wt. and 2.1 mM/Kg wet wt. Fig. 9 shows clearly that while IAA induced a net Ca gain it had no effect on the rate of Ca^{45} efflux. Since the net Ca uptake during efflux would increase the dilution of Ca^{45} by Ca^{40} in the IAA treated horns over the controls the Ca efflux was probably raised during inhibition.

These experiments lead to the conclusion that the IAA induced net Ca gain is caused by an increase in the Ca influx and not by a decreased efflux. Thus the Ca gradient across the membrane is maintained

Fig. 10. Typical experiment done on two uterine horns from the same rat showing that IAA induced net Ca uptake is not accompanied by a change in Ca^{45} efflux. The experimental horn (X) was loaded in Ca^{45} labelled K.R. for 80 minutes and in labelled K.R. containing 1 mM IAA for an additional 40 minutes. It then was effluxed into K.R. containing 1 mM IAA. The control horn (.) loaded and effluxed for the same times was not exposed to IAA. The curves on the left represent the rate of efflux vs time, and those on the right Ca^{45} tissue content vs time. For details on uptake, see text.


m Moles / kg wet
22
20
18
16
14
12
10
8
6
4
2
25
50
75
100
200
300
400
500
Time in minutes

by a metabolism dependent exclusion mechanism.

Effect of IAA on the net Ca uptake from Tris buffered solution containing 10 mMolar Ca.

Uterine horns were incubated for various times in T.S. containing 10 mMolar Ca, and in T.S. containing 10 mMolar Ca plus 1 mMolar IAA. As can be seen in Fig. 11 the curves for the IAA treated control tissues are almost the same for the first hour. They show an initial fast uptake of about 4 mM/kg wet wt. which is the amount expected for equilibration with the extracellular space. Then the tissue Ca concentration rises more slowly to an almost steady value of close to 7 mM/kg wet wt., which is maintained for about 30 minutes in the IAA treated, and for about one hour in the control tissues. Calculations show that at a tissue Ca concentration of 7 mM/kg wet wt. and an external Ca concentration of 10 mMolar the cellular Ca concentration (including inexchangeable and bound Ca) is about 4 mM/liter cell water. The equilibrium value for intracellular Ca concentration is however 500 mMolar (assuming V equals -50 mV). Thus 7 mM Ca/kg wet wt. is a

Fig. 11. Uptake of total Ca by rat uteri from T.S. containing 10 mMolar Ca and 1 mMolar IAA (X) and from same without IAA (·). For times 10 and 20 minutes crosses omitted since values overlapped. Vertical bars indicate twice S.E. of 4 determinations. In absence of bars twice the S.E. is less than the diameter of the points. (●), (O) Obtained from progesterone dominated uteri (see text) at external Ca concentration of 10 mM with and without 1 mMolar IAA respectively.

temporary steady state level at which the exclusion pump is still effectively keeping Ca out of the cells. The effect of IAA is then seen to be a delayed net Ca uptake of about 14 mM/kg wet wt.

At 21 mM Ca/kg wet wt. the cellular Ca concentration would still be far below the equilibrium value if the potential were still -50 mV. At an external Ca concentration of 10 mM and in presence of a normal membrane potential electrochemical equilibrium would demand an intracellular Ca concentration of 490 mMolar. However there are convincing reasons to believe that prolonged IAA treatment abolishes the membrane potential (see pg. 51) and under these circumstances 11 mM Ca/kg wet wt. represents bound Ca (see section on binding). Although more delayed and slower, the Ca concentration in the control tissues rises to the same final concentration of about 21 mM/kg wet wt.

One of the most obvious explanations for the delay in the action of IAA is that during the delay period the store of ATP is being exhausted. Only after this has occurred does the Ca exclusion pump begin to fail resulting in a net Ca uptake. Using a similar argument the latent secondary rise of Ca concentration in the control tissues can be explained as follows. The unusually high external Ca concentration places excessive demand on the energy (in the form of ATP) available for active transport. Since it is assumed that the metabolic energy supply is not inhibited in the absence of IAA, but only that the demands are excessive it follows that a longer period of time is necessary for the ATP levels to be reduced sufficiently to interfere with active ion transport than in the case of IAA. Also since metabolism is still taking place during the secondary Ca gain this gain would be expected to be slower than in the IAA treated uteri. This theory predicts

that prolonged exposure to 10 mMolar Ca will also cause a net downhill movement of Na and K. For this reason a number of uteri were exposed to T.S. containing 10 mMolar Ca for five hours. They were then blotted, wet ashed by heating to 200 degrees C in nitric acid (conc.) for several days, and then analysed for Na and K using the same internal standard technique as described for Ca. The results showed a large net downhill movement for potassium to 13 mM/kg wet wt. ± 1.2 (5), and for sodium to 104 mM/kg wet wt. ± 3.5 (5). The normal K concentration is about 75 mM/kg wet wt. The external Na concentration in these experiments using T.S. is 115 mMolar, so that the sodium space increased to 900 ml/kg wet wt. Large downhill ion movements in the uterus are accompanied by an increase in water content to about 900 ml/kg wet wt. (8), so that the intracellular Na became equal to the external Na concentration. The membrane potential is dependent on the K gradient across the cell membrane and perhaps electrogenic ion transport. Thus large downhill ion movements and inhibition of ion transport in the controls probably also led to complete membrane depolarization. The lower circle in Fig. 10 indicates that the secondary net Ca uptake occurs more slowly in progesterone dominated uteri than in estrogen dominated ones.*

Effect of cold on Ca concentration.

Table 1. demonstrates that 24 hours at 4 degrees C does not

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* Values are averages from 2 rats treated 2 days with 100 μgm diethylstilbesterol, followed by 1 day 100 μgm diethylstilbesterol plus 50 μgm progesterone, followed by 2 days 50 μgm progesterone and killed on the last day of treatment, and 4 older rats (120 mg) treated 3 days with 100 μgm diethylstilbesterol and left untreated for 2 more days.

raise the uterine Ca concentration.

Table 1. Total Ca concentration of rat uteri incubated in K.R. at 4° C.

Time at 4° C in hours	Total Ca mM/kg wet wt. by flame photometry	Total Ca mM/kg wet wt. Ca^{45} method see text
0	2.0 ± 0.19 (4)	2.1 ± 0.11 (5)
15	2.4 ± 0.09 (5)	2.2 ± 0.04 (5)
24	2.1 ± 0.04 (5)	2.3 ± 0.08 (5)
36		2.6 ± 0.04 (5)

Effect of ouabain on Ca concentration.

Rat uterine horns were incubated in K.R. containing 1 mMolar ouabain for 1, 2, and 6 hour periods and then analyzed for total Ca concentration. Uteri serving as controls were incubated for the same periods in K.R. Table 2. demonstrates that ouabain does not cause a net uptake of total tissue Ca.

Table 2. Total Ca concentration of rat uteri incubated in K.R. containing 1 mMolar ouabain.

Time in hours	Total Ca mM/kg wet wt. Controls	Total Ca mM/kg wet wt. Ouabain treated
1	2.0 ± 0.13 (5)	2.0 ± 0.07 (4)
2	2.1 ± 0.20 (3)	2.1 ± 0.08 (8)
6	2.3 ± 0.07 (3)	2.2 ± 0.15 (4)

Effect of varying the external Na concentration on total Ca.

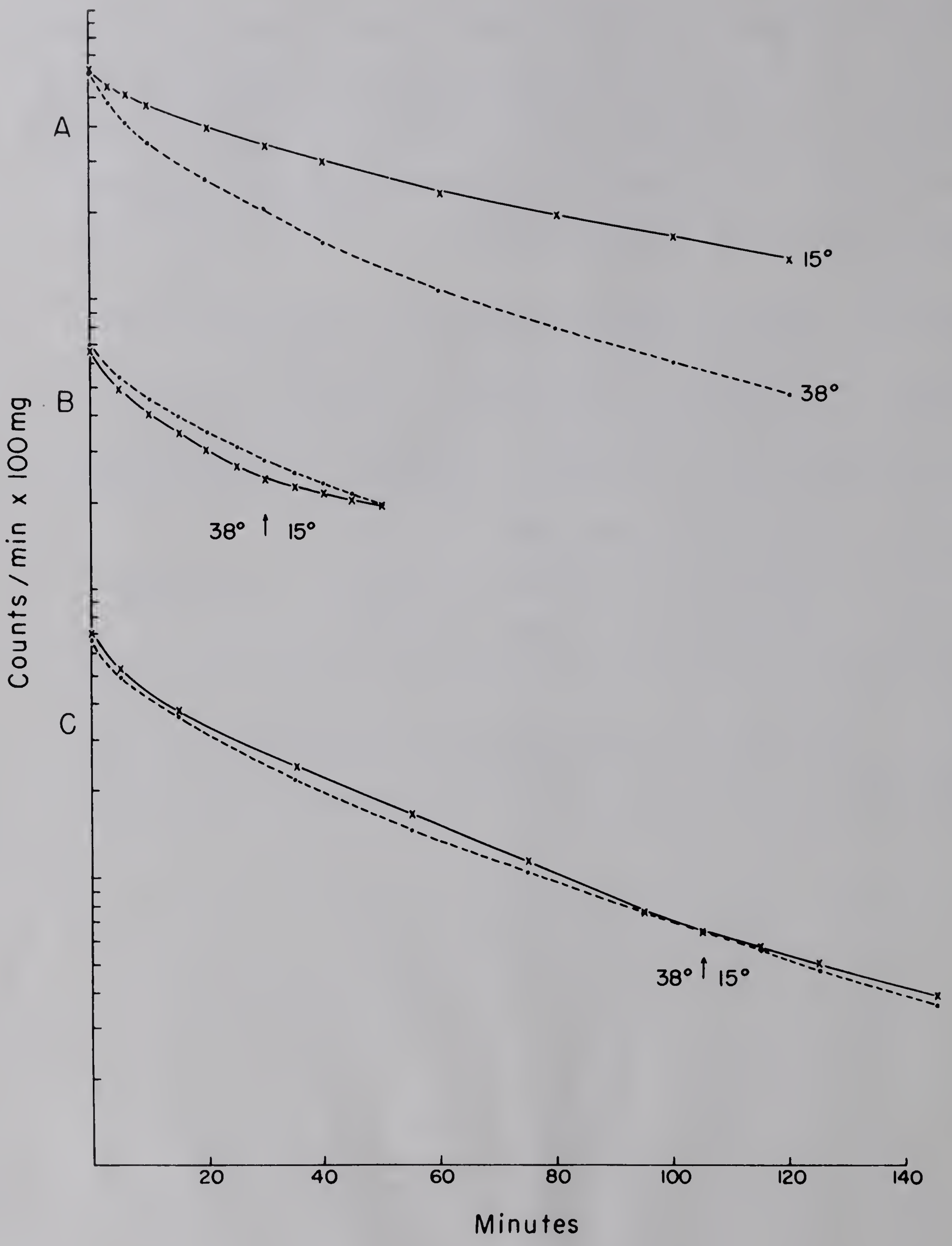
To examine a possible competition between Na and Ca for membrane transport sites the Na concentrations were varied by adding

NaCl and substitution of NaCl by equimolar amounts of Choline Cl in K.R. and T.S. Rat uterine horns were incubated in these media for 10, 60 and 120 minutes and analyzed for total tissue Ca by both flame photometry and the Ca^{45} method. Since the results of both methods were the same their averages are presented in Table 3.

Table 3. Effect of varying the external Na concentrations on the total Ca concentration of rat uterine horns.

Time minutes	External Na concentrations of incubation solutions			
	10 mMolar	50 mMolar	200 mMolar	138 mMolar (K.R.)
10	2.2±0.21(4)	2.2±0.14(6)	2.0±0.11(4)	
60	2.3±0.23(4)	2.3±0.20(4)	2.3±0.11(4)	2.1±0.11(5)
120	2.2±0.15(5)	2.3±0.10(5)	2.3±0.09(4)	

The results show that between 10 and 200 mMolar the Na concentration has no effect on the total Ca concentration of the uteri.


A
15°
38°
B
38° ↑ 15°
C
38° ↑ 15°
Counts / min x 100 mg
20
40
60
80
100
120
140
Minutes

Effect of temperature on Ca^{45} efflux.

To determine the effect of cold the temperature was lowered from 38°C to 15°C at three different times during efflux. All uterine horns were loaded at 38°C for 2 hours and the experimental horns were either effluxed at 15°C for 2 hours (Fig. 12 A), at 38° for 30 minutes followed by 15°C for 20 minutes (Fig. 12 B), or at 38° for 105 minutes followed by 15°C for 40 minutes. (Fig. 12 C). Fig. 12 shows one of 2 such experiments, which both showed a relatively small temperature effect on Ca^{45} efflux.

Q_{10} values were calculated from the change in slope of the experimental curves in B and C and from the difference in slope between the experimental and control curves at times 30 minutes and 105 minutes in A. The Q_{10} at 30 minutes was 1.35 in both cases and at 105 minutes was 1.26 for A and 1.29 for C.

These low Q_{10} values implicate a purely physical process for Ca^{45} efflux.

However sudden lowering of temperature will cause membrane depolarization and contracture (28) (uteri were observed to contract upon lowering the temperature in these experiments) and these might be complicating factors.

Fig. 12. Ca^{45} efflux at 15°C A (X). Temperature was lowered during efflux from 38°C to 15°C at the arrows in B and C for the experimental uteri (X). Control efflux curves at 38°C (·) were obtained from uterine horns from the same rats as the experimental ones.

CALCIUM BINDING

METHODS

Equilibration of tissue Ca with external Ca concentration.

Uterine horns dissected as usual were placed in T.S. containing 1 mMolar IAA and varying Ca concentrations for 330 minutes. They were then blotted, ashed and analyzed for total Ca concentration.

The same experiment was repeated with 2,4-dinitrophenol (DNP), replacing IAA.

Measurement of resting membrane potential after 330 minutes in IAA.

The uteri were mounted serosal side upward, by spreading and pinning on a wax surface in the bottom of a small jacketed constant temperature bath at 37 degrees C. Two uteri were simultaneously mounted in this manner in two adjoining baths, one containing K.R. and the other containing K.R. with 1 mMolar IAA. The experimental tissues were exposed to 1 mMolar IAA for 5 hours prior to the membrane potential determinations. Single cell electrical activity of the experimental and control uteri was recorded by means of handpulled glass micro-electrodes filled with 3 Molar KCl using the floating electrode technique of Woodbury and Brady (9). The electrode tips were inspected under a microscope. Only electrodes whose tips did not leak and had a resistance greater than 20 mega-ohms were used. Potential measurements were made through a Medistor negative capacitance electrometer, monitored on a Tektronix 502 oscilloscope and recorded on a Grass polygraph.

RESULTS

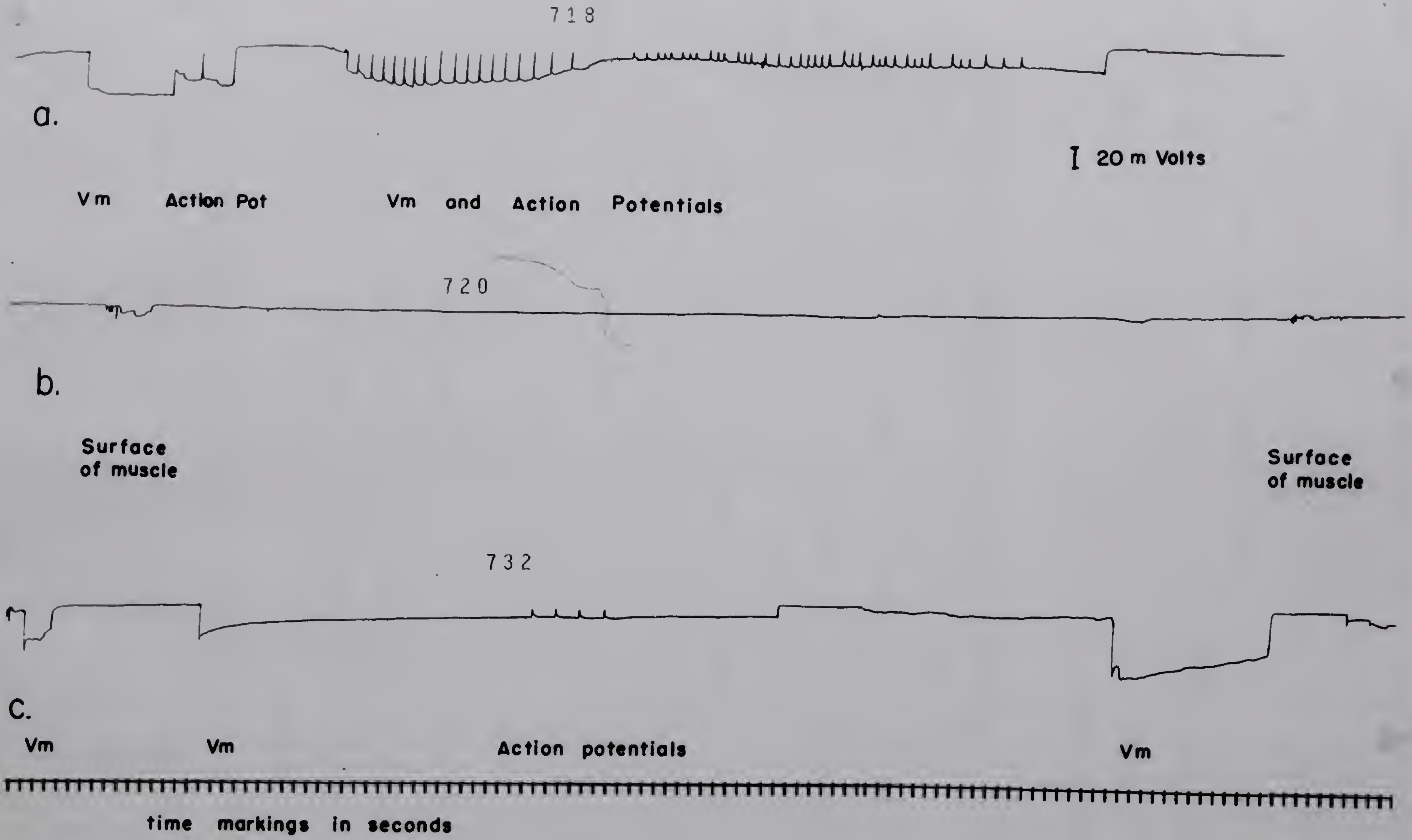
At a steady state for Ca, the tissue Ca concentration is determined by the external Ca concentration, the membrane potential, active Ca exclusion and cellular binding. Since active exclusion pre-

vents equilibration of the intracellular with the external Ca, Ca binding can not be studied by simply varying the external Ca concentration and measuring the tissue Ca concentration. If the active transport of ions is inhibited with 1 mMolar IAA for a sufficient period to reach Ca equilibrium across the cell membrane, and if the membrane potential can be estimated, then values necessary for quantitative treatment of cellular Ca binding can be obtained.

Estimation of Membrane potential after 5 hours of IAA treatment.

Daniel (8) reported that 3 hours exposure to 1 mMolar IAA reduced the K concentration to 67 meq/kg dry wt., and increased the Na concentration to 1237 meq/kg dry wt. and the tissue water to 887 ml/kg wet wt. This means that the K concentration was 8.6 meq/liter tissue water compared to 5.8 mMolar in the bathing solution, and the Na concentration 158 meq/liter tissue water, compared to 145 mMolar in the bathing solution. These values indicate that the K and Na gradients are completely abolished, the small excess of ions being due to a lower activity coefficient in tissue water, to binding or to both. Since the two immediate causes of membrane potential are ionic gradients and electrogenic ion transport and since IAA abolishes both, it would be expected that a 5 hour exposure to 1 mMolar IAA would also abolish the membrane potential.

During attempts at direct determinations of membrane potential of rat uterine smooth muscle a resting membrane potential of about 40 mV could be frequently recorded in the control tissues, but no detectable membrane potential could be obtained from the IAA treated ones. Some of these results are shown in Fig. 13. It is clear that genuine penetrations and potential recordings were made and that if the IAA


718
a.
20 m Volts
Vm
Action Pot
Vm and Action Potentials
720
b.
Surface of muscle
Surface of muscle
732
c.
Vm
Vm
Action potentials
Vm
time markings in seconds

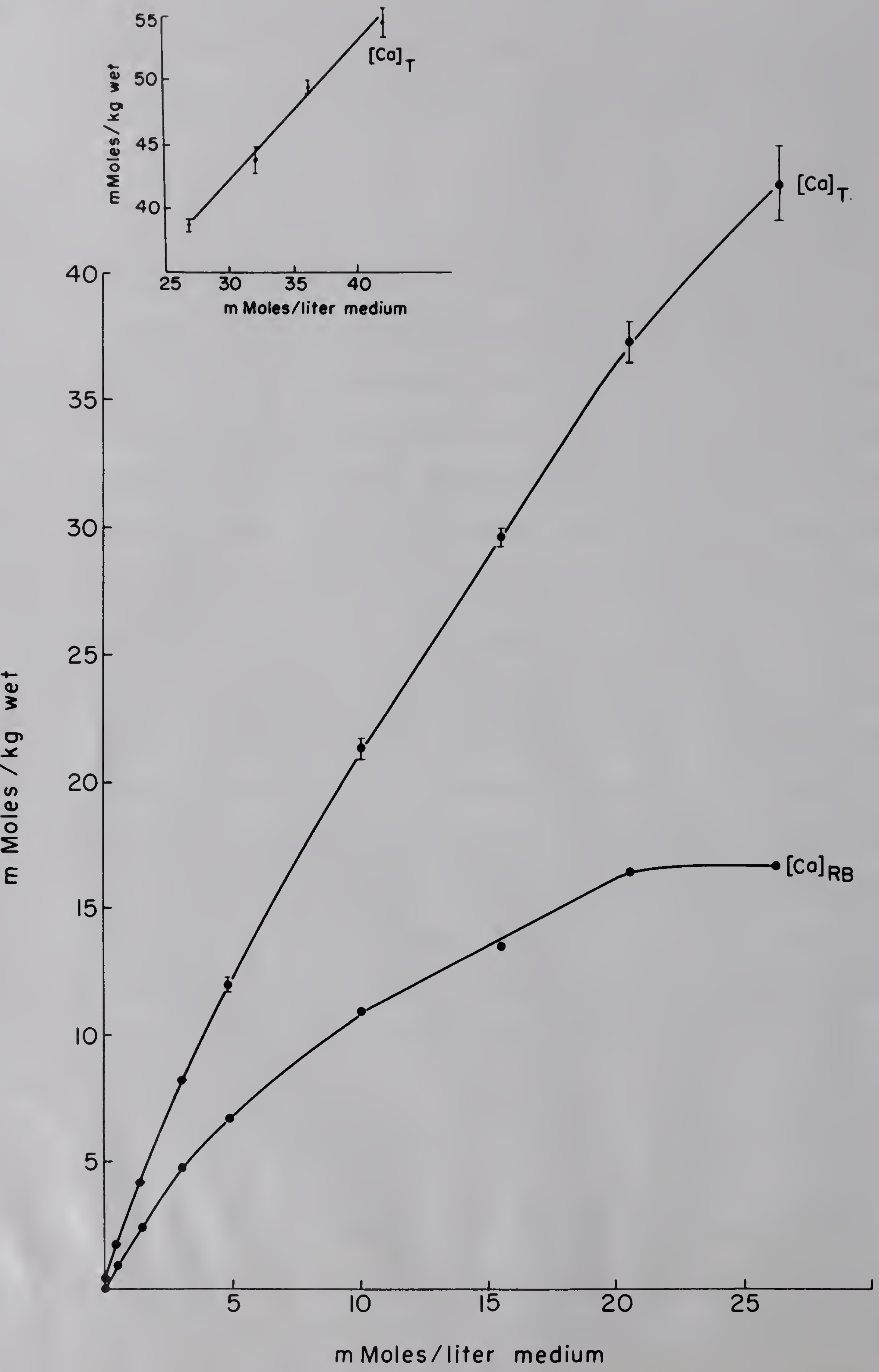
treated cells did have membrane potentials these should have been much easier to record, as IAA swells the cells and inhibits spontaneous contractions.

Uterine Ca binding.

The data presented and quoted above have established with reasonable certainty that after 330 minutes exposure to IAA the two important factors determining tissue Ca concentration are the external Ca concentration and Ca binding. Thus uteri were incubated for 330 minutes in T.S. containing 1 mMolar IAA and varying Ca concentrations. The relationship between the Ca concentration in the tissues after this treatment and the Ca concentration of the T.S. is illustrated in Fig. 14. The slope of this curve decreases continually until at Ca concentrations higher than 20 mMolar it remains constant at a value of one. This final slope of one was confirmed as shown in the inset of Fig. 12 in an experiment using a different group of rats and higher Ca concentrations. The best explanation for this direct proportionality between the external and tissue Ca concentrations is that at an external Ca concentration of 20 mMolar all the tissue binding sites are occupied so that when the external Ca concentration is raised beyond this value the increase in tissue Ca is due solely to an increase in ionic Ca.

In agreement with Daniel's results (8) the tissue water in

Fig. 13. Intracellular potential recordings from uterine smooth muscle. a and c are controls recorded before and after recording b from uterine smooth muscle treated with 1 mMolar IAA for 5 hours. These recordings were made using the same micro-electrode, which had a resistance of 40 mega-ohms.

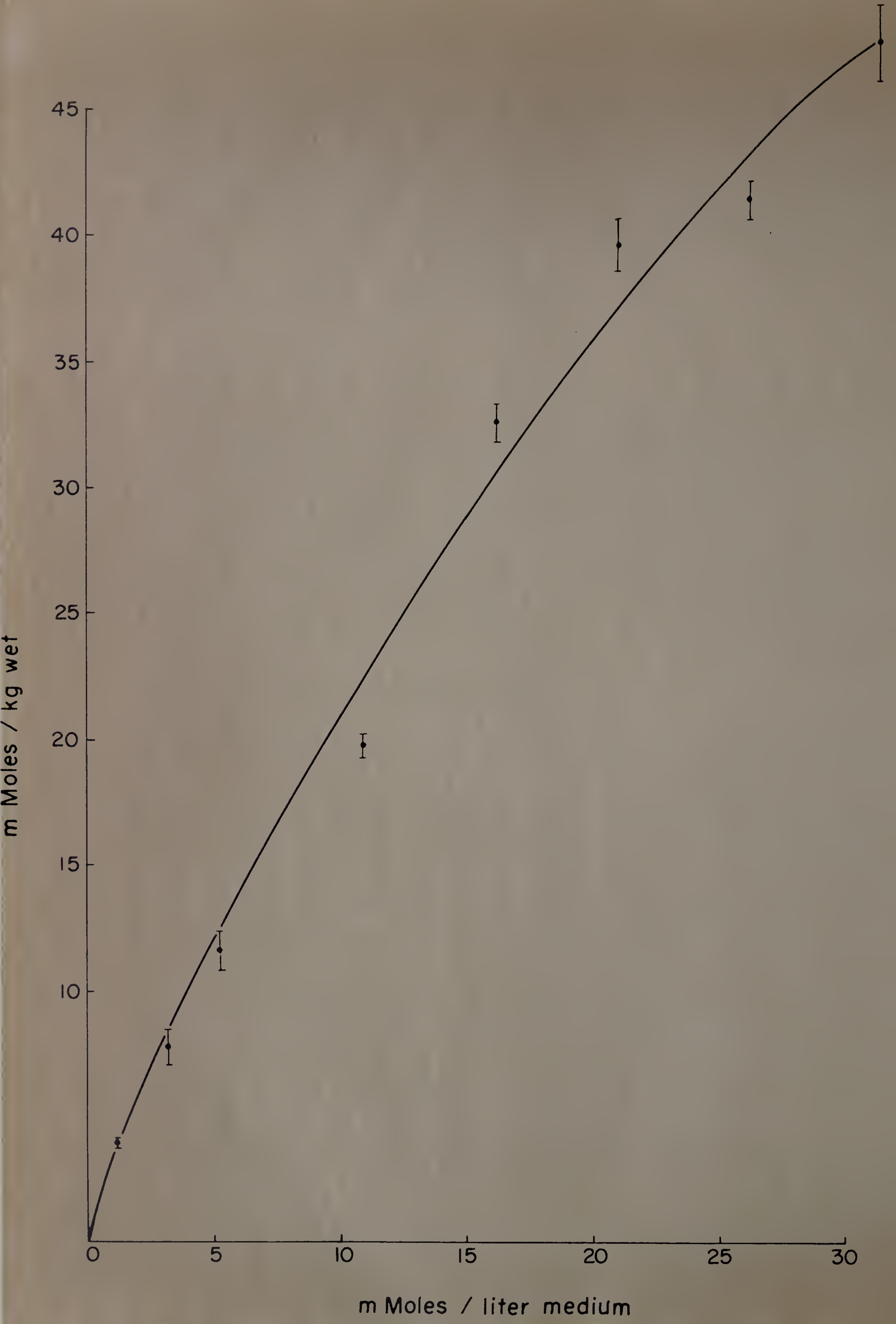

55
50
45
40
mMoles/kg wet
25
30
35
40
m Moles/liter medium
[Ca]$_T$
40
35
30
25
20
15
10
5
m Moles / kg wet
5
10
15
20
25
m Moles/liter medium
[Ca]$_T$.
[Ca]$_{RB}$

this case was 870 ml/kg wet wt., so that at external concentrations above 20 mMolar the extra tissue Ca in mMoles/liter tissue water was 1.16 times the increase in the external solution. Such a relationship would be explained if tissue ionic Ca has an activity coefficient of 0.87.

The observation that after prolonged incubation in IAA the ionic tissue Ca in mM/kg wet wt. is numerically equal to the external Ca concentration in mMoles/liter allows calculation of the reversibly bound Ca by subtraction of the ionic and .45 mM/kg wet wt. inexchangeable Ca from the total tissue Ca concentration. The relationship between the reversibly bound Ca and the external Ca so obtained is shown in the lower curve of Fig. 14. The slope of this curve becomes zero when all the Ca binding sites are occupied. At this point the bound Ca equals the total number of binding sites.

Since as pointed out above no evidence was found for extensive extracellular binding, the binding sites shown to exist by these experi-

Fig. 14. The upper curve gives the variation of tissue Ca concentration with the external Ca concentration. These values were obtained from rat uteri incubated in T.S. containing 1 mMolar IAA for 330 minutes. The inset shows the same for higher Ca concentration. The lower curve shows the relationship between external Ca concentration and the Ca which is reversibly bound by the tissues under the same conditions. See text for calculation of this curve. Each point is average of 4 determinations. Bars equal 2 times S.E. Where bars are absent 2 times S.E. is less than the diameter of the dots. No. S.E.'s are indicated in lower curve.


45
40
35
30
25
20
15
10
0
5
10
15
20
25
30
m Moles / kg wet
m Moles / liter medium

ments are assumed to be located mainly intracellularly and have a concentration of 16.75 mM/kg wet wt. The concentration of free sites can now be calculated by subtracting the bound Ca from the total number of binding sites.

To test if the observed binding was specifically affected by the IAA, the experiment was repeated using DNP as a metabolic inhibitor instead of IAA. Fig. 15 shows that the curve obtained with IAA fits the points obtained with DNP.

Analysis of the data on binding showed that it did not follow a simple mass action law. The results will first be treated as if a simple association constant for the observed Ca binding exists. Then an explanation will be given for the observed deviation from the simple mass action law.

If Ca binding occurred by one specific molecule Pr and each molecule Pr would bind only one Ca ion, then the association constant for Ca binding would be given by: $K = \frac{CaPr}{Ca.Pr} \cdot \frac{f_{CaPr}}{f_{Ca} \cdot f_{Pr}}$

K = assoc. constant

CaPr = molar conc. of CaPr complex

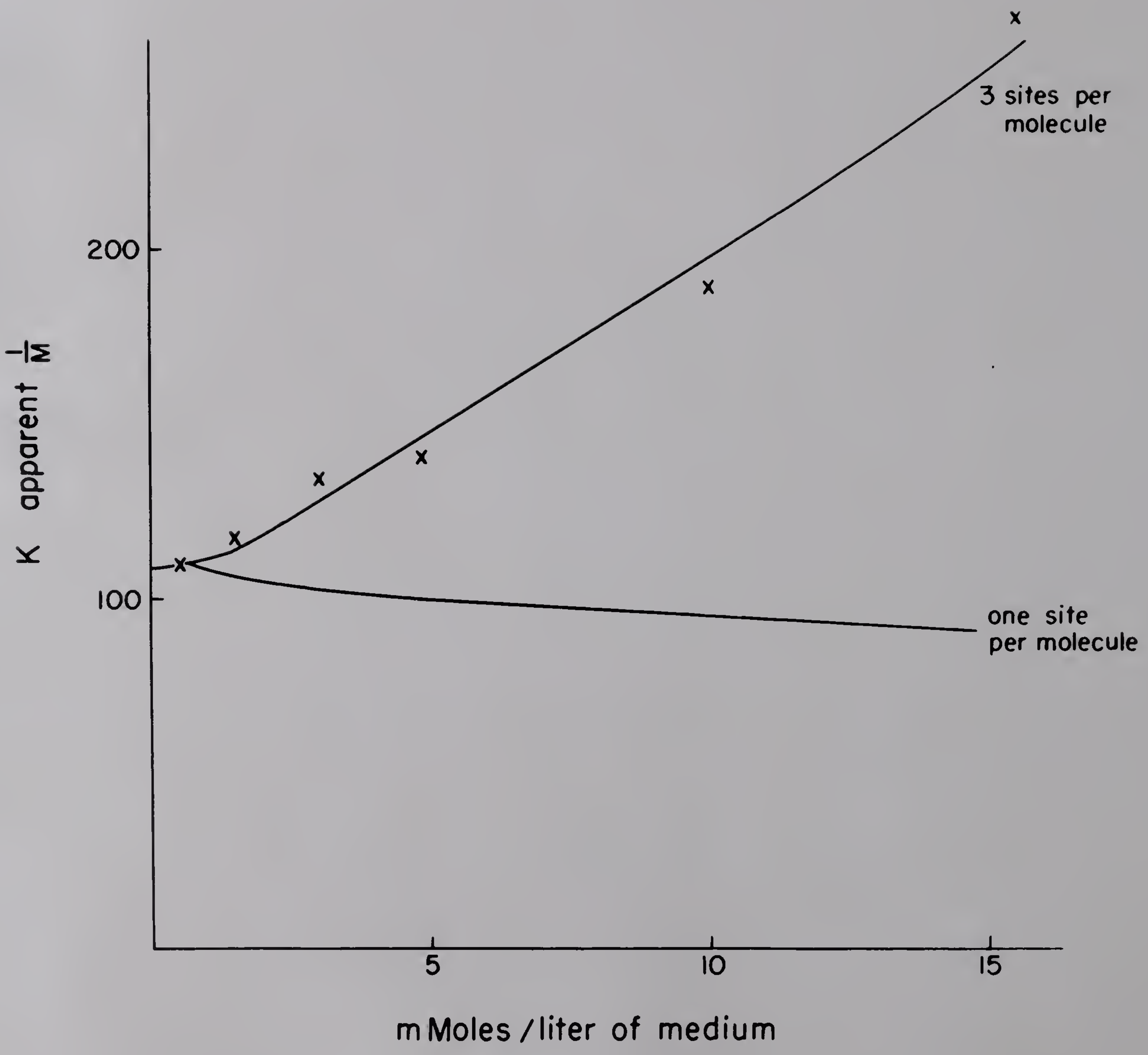
Ca = molar conc. of Ca ions

Pr = molar conc. of Pr

f = activity coefficient of molecule or ion in subscript..

If the activity coefficients of CaPr and Pr do not differ

Fig. 15. Total Ca concentration of rat uteri incubated for 330 minutes in T.S. containing 1 mMolar 2,4-dinitrophenol and varying Ca concentrations. Each point is an average of 4 determinations. Bars equal 2 times S.E. Note that curve drawn to fit points is identical to that in fig. 14.



K apparent $\frac{1}{M}$
200
100
5
10
15
mMoles / liter of medium
3 sites per molecule
one site per molecule

appreciably the above association constant is approximated by:

$$K\ app. = \frac{Ca\ rev.\ bound}{Ca\ ext.\ x\ sites\ free}$$

The values calculated for the apparent association constant at each of the external Ca concentrations below 20 mMolar are given by the corsses in Fig. 16. The theoretical curve fitted to these experimental points was calculated using the equations developed by Klotz (10) for the case of ion binding by protein having a number of different binding sites per protein molecule. Thus the theoretical curve satisfies the following conditions:

$$r = \frac{K_1Ca + 2K_1K_2Ca^2 + 3K_1K_2K_3Ca^3}{1 + K_1Ca + K_1K_2Ca^2 + K_1K_2K_3Ca^3}$$

r is concentration of bound Ca/concentration of total Pr., K_1 is the association constant for binding of Ca ions with the first binding site, K_2 and K_3 the same for the second and third binding sites, Ca is the external Ca concentration. The values used for K_1, K_2, and K_3, chosen to give the best fit were respectively 330, 100, and 160 1/molar. Using the saturation value of 16.75 mM.Kg wet wt. divided by 3 sites/molecule for total Pr., the calculated values of r, and the external Ca concentrations, the theoretical K apps analogous to the experimental ones were then

Fig. 16. Crosses indicate the apparent equilibrium constants calculated according to the mass action law from the lower curve in Fig. 14. The curve fitted to these points was calculated according to equations developed by Klotz (see text) assuming three binding sites per Ca binding molecule with association constants 330, 100, and 160 respectively. The lower curve illustrates the case where Ca binding satisfies two equilibrium constants for the interaction between Ca and two different kinds of molecules each binding only one Ca ion per molecule.

calculated. Note that these apparent association constants increase with increasing external Ca concentrations. The lower curve with the negative slope represents theoretical K apps if binding occurred by two different proteins each binding only one Ca ion per protein molecule. The slope would also be negative if there were more than two kinds of such proteins.

The association constants 300, 100 and 160 fall within the range of association constants reported for serum proteins by Greenberg (11).

The conclusion from these experiments is that under conditions of metabolic inhibition, Ca is bound by cellular proteins containing more than one binding site per protein molecule. The significance of this binding will be considered in the discussion.

EFFECTS OF HIGH POTASSIUM DEPOLARIZATION AND ACETYLCHOLINE ON CALCIUM EXCHANGE

METHODS

Solutions.

High K depolarizing solution: mM/liter K 191, Na 21.9, Ca 1.5, Mg 1.0, Cl 4.63, HCO_3 21.9, H_2PO_4 1.12, SO_4 94, glucose 50. (191 K).

Medium K depolarizing solution: mM/liter K 40, Na 103, Ca 1.5, Mg 1.0, Cl 123, HCO_3 21.9, H_2PO_4 1.12, SO_4 1, glucose 50. (40 K).

These solutions were bubbled with 95% O_2 and 5% CO_2. Temperature 37 degrees C.

The solutions were labelled with Ca^{45} where necessary as indicated in the results.

Acetylcholine Bromide was added to some solutions as indicated in the results.

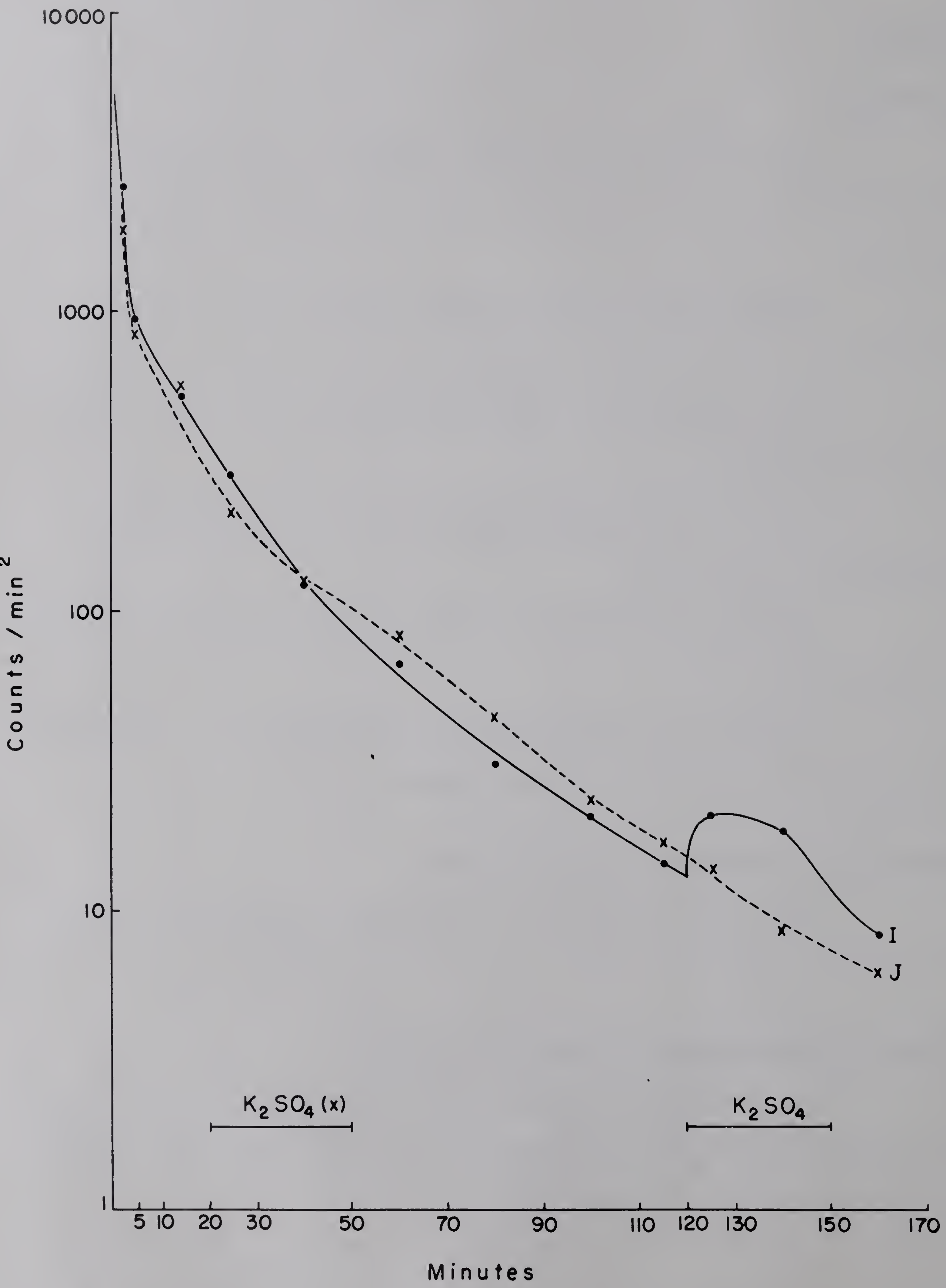
Ca free K.R. is simply K.R. with $CaCl_2$ left out. One Ca free solution had in addition 0.7 mMolar Na_3EGTA.

Effects of different media on Ca^{45} efflux.

These were determined by substituting the different media for K.R. in the efflux tubes over the time intervals indicated under results.

Effects of different media on the Ca^{45} influx.

The procedure is identical with the control Ca^{45} influx except that different Ca^{45} labelled media are used, details about the sequences of media will be given under results.


10000
1000
100
10
1
Counts / min ^{2}
5 10 20 30 50 70 90 110 120 130 150 170
Minutes
K_2SO_4 (x)
K_2SO_4
I
J

RESULTS

Effect of 191 mMolar K on Ca^{45} efflux.

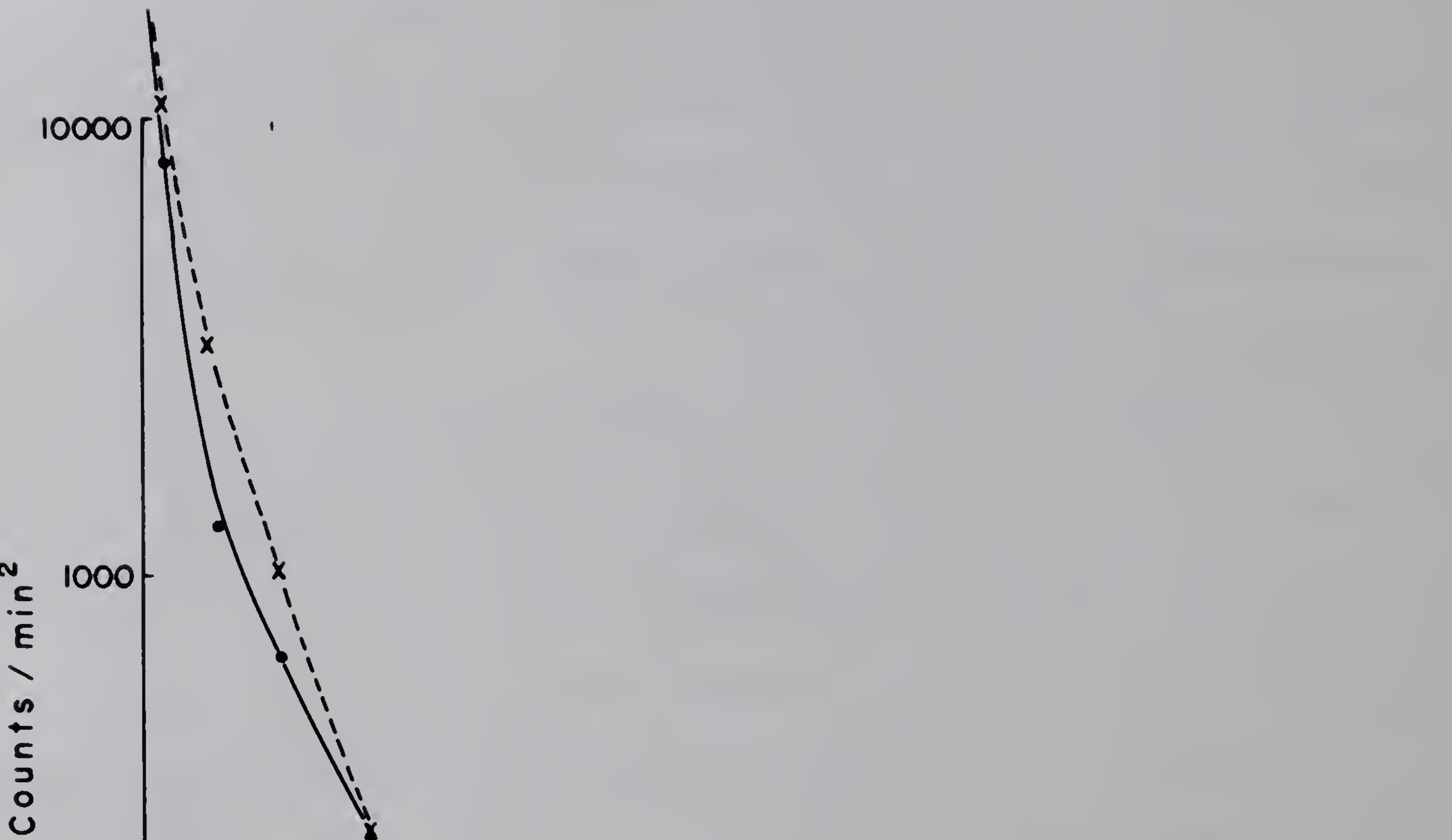
Uterine horns were incubated in Ca^{45} labelled K.R. for 2 to 3 hours before starting the efflux in K.R.. This period is sufficient to label practically all exchangeable Ca with Ca^{45}, see section 1. After effluxing in K.R. for the first 20 minutes the tissues were transferred to tubes containing 191 K for a total of 30 minutes, after which time they were again passed through a series of tubes containing K.R.. Whenever the uteri were exposed to 191 K they contracted vigorously and remained contracted forthe entire high K exposure period. Fig. 17 shows the typical result of this procedure, namely high K depolarization has no effect on the early part of the Ca^{45} efflux. High K depolarization causes a rise in the rate of efflux only if the depolarizing solution is applied after the tissues have been allowed to efflux into K.R. for about 2 hours. This result is also illustrated in Fig. 17.

The observed increase in efflux is not likely to be due to an

Fig. 17. Effect of high K depolarization (191 K) on the Ca^{45} efflux from rat uteri previously loaded with Ca^{45} for 2 to3 hours. The high K solution was substituted for K.R. as efflux medium during the times indicated by both horizontal bars for one uterine horn (X), and for the period indicated by the second horizontal bar for the other uterine horn from the same rat (.). The curves show that high K concentration causes a rise in the rate of efflux only late in the efflux period, and that this rise is prevented by a previous depolarization early during the efflux period as shown by the dashed curve.

increased membrane permeability for Ca, or easier membrane permeation outward due to reversal of the electrical driving force on Ca ions. In these cases the efflux of Ca^{45} should have been enhanced also after 20 minutes of efflux into K.R., since at this time nearly all the extracellular Ca should have exchanged and thus most of the observed efflux was due to cellular Ca^{45}. However the effect of high K depolarization was not observable until the efflux of cellular Ca^{45} had fallen to a very low level. This suggests that the effect of high K depolarization did occur early during efflux,but was obscured by the large efflux of intracellular Ca^{45}.

At this point it is most convenient to postulate a working hypothesis for the source of Ca involved in the high K induced increase in Ca^{45} efflux. Results of experiments designed to test the hypothesis will then be presented. It is proposed that the high K depolarization releases Ca from the cell surfaces, and that this superficial Ca fraction is small; is in parallel with the intracellular Ca, and exchanges very slowly. Such a fraction would have the following properties: (1) Due to the small size of the fraction the release of Ca^{45} from it would be obscured by a large efflux of intracellular Ca^{45} during the early part of the efflux period. (2) The release could be observed during the latter part of the efflux period, since the specific activity of the intracellular Ca would decrease more rapidly than that of the superficial fraction in question. (3) As this superficial fraction is in parallel with the cellular Ca once its Ca^{45} has been released it would not be restored by exchange with Ca^{45} leaving the cells. (4) Its Ca should be depleted by the use of an extracellular Ca chelating agent such as EGTA. (5) Its presence should be demonstrated in the isolated smooth

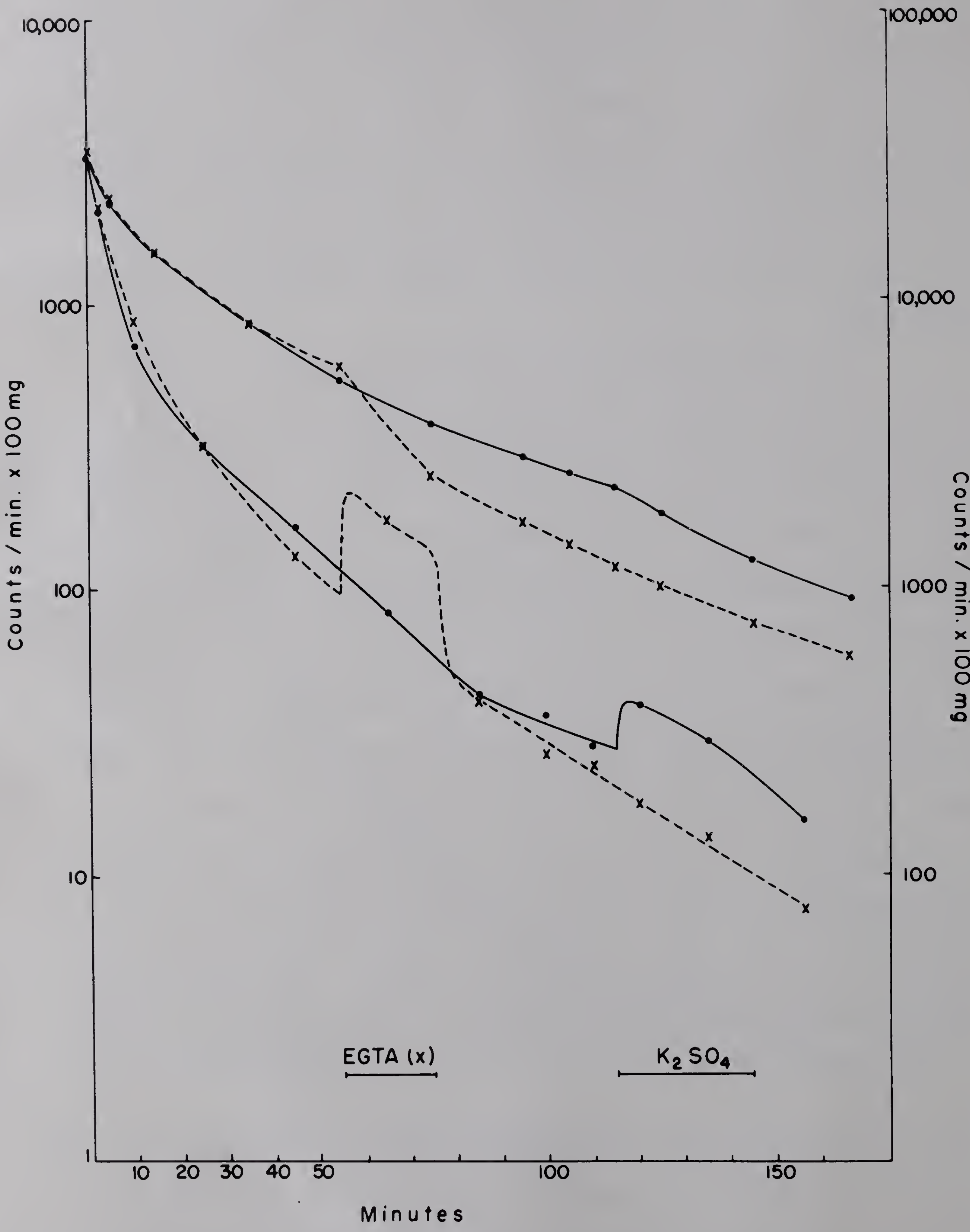

10000
1000
100
10
Counts / min^2
10
20
30
40
60
80
100
120
140
Minutes

muscle preparation. (6) Due to the slow exchange of this fraction a short incubation period should be insufficient to load it with Ca^{45}.

The experimental results illustrated in Fig. 17 demonstrate the first three properties. The dashed curve is from a uterus which was exposed to 191 K after 20 minutes and again for another 30 minute period after 2 hours. Although the first exposure to 191 K does not appreciably lower the Ca^{45} content below that of the control uterus, it abolished the increased efflux on the second exposure. The uterus did completely relax after the first exposure and contract again on the second one.

Fig. 18 shows that when the endometrium plus most of the circular smooth muscle is stripped away from the longitudinal smooth muscle both parts of the uterus exhibit the high K induced increased efflux to a more marked extent than does the whole uterus. In a number of experiments performed on dissected longitudinal muscle the high K induced increased efflux was very large (up to 10 times) and variable. Due to the probability of a contribution to this effect by the extra Ca associated with tissue damage only the results obtained from whole uteri will be presented in the further analysis of this phenomenon. In the experiment illustrated in Fig. 19 the effects of an initial 20 minute exposure to Ca free K.R. containing 0.7 mMolar EGTA on the Ca^{45} efflux and the final depolarization were tested. This procedure was compared with a normal efflux followed by late application of 191 K,

Fig. 18. The Ca^{45} efflux from dissected uterine smooth muscle (·) and from the remainder of the uterine horn (X) (see text for details of dissection) into K.R. followed by a final 20 minutes (120-140) into 191 K.

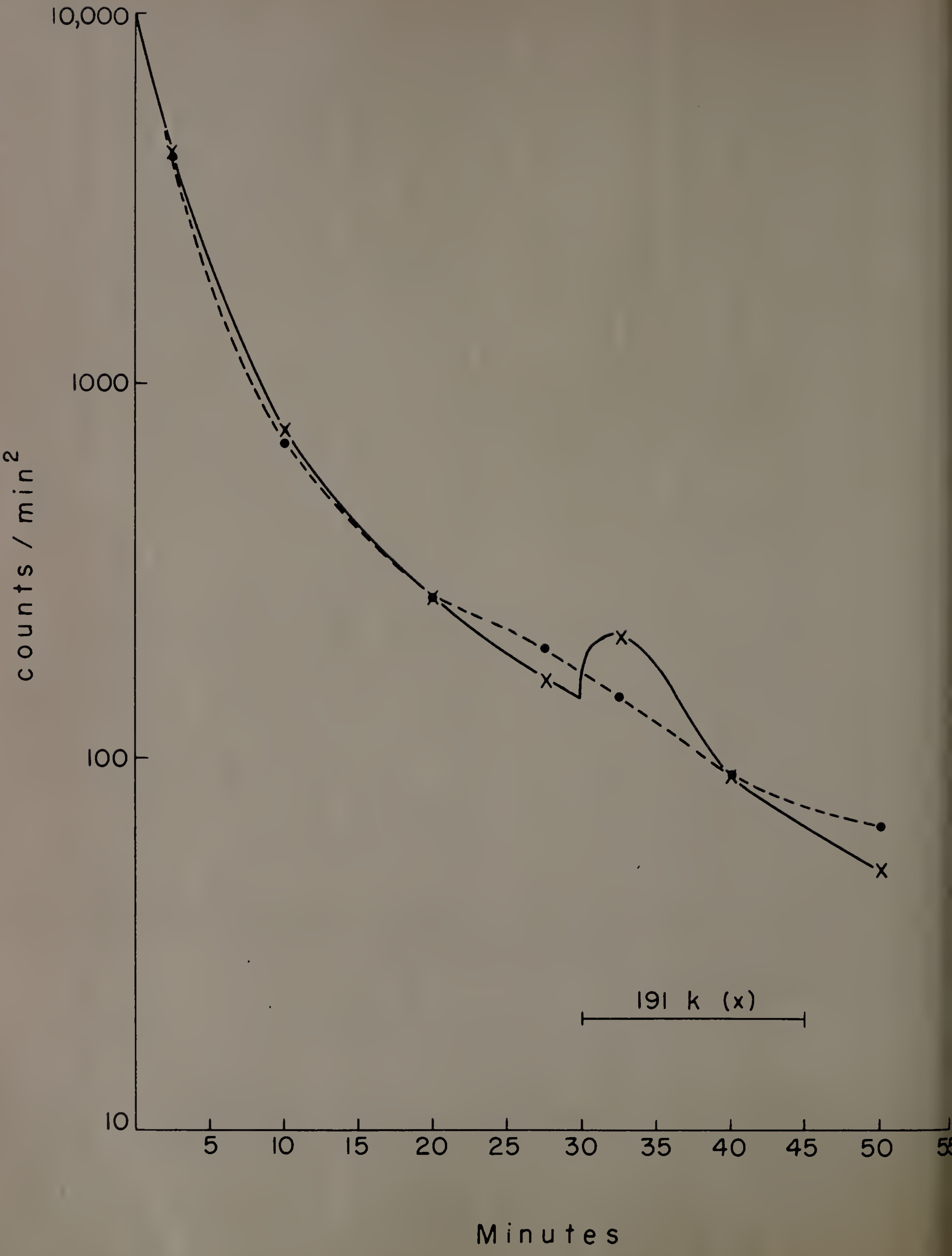

10,000
1000
100
10
1
Counts / min. x 100 mg
100,000
10,000
1000
100
Counts / min. x 100 mg
10
20
30
40
50
100
150
Minutes
EGTA (x)
K_2SO_4

done on the paired uterine horns from the same rats. The results are shown as Ca^{45} in the tissues vs time in the upper curves, and as the rate of efflux vs time in the lower curves. The Ca concentration of K.R. and 191 K used in this experiment was raised to 2.5 mM*.

The results show that chelation of the extracellular Ca by EGTA increases the rate of Ca^{45} efflux, and that it also removes the Ca which is involved in the increased efflux induced by high K depolarization. The rate curves show that although immediately before depolarization the efflux rate of the experimental horns is close to that of the controls the depolarization has no effect on the rate of efflux in the EGTA treated uteri. Finally the 6th point was tested by incubating uteri in Ca^{45} labelled K.R. for only 10 minutes. The tissues were then effluxed into K.R. for 30 minutes, in 191 K for 15 minutes and for a final 10 minutes into K.R. Fig. 20 shows the average results

* At 2.5 mMolar Ca the response to 191 K was more uniform than at the usual concentration of 1.5 mMolar, at which concentration it occasionally was so small as to be difficult to analyze.

Fig. 19. The dashed curves show the Ca^{45} efflux from uterine horns for successive periods into K.R., into Ca free K.R. containing 0.7 mMolar EGTA, into K.R., into 191 K, and finally into K.R. Opposite horns from the same rats were effluxed in K.R. and 191 K only. The times of EGTA and high K exposure are indicated by the horizontal bars. The upper curves indicate the loss of radioactivity and the lower curves the rate of loss of radioactivity from the tissues. Each point is an average of 5 determinations.


10,000
1000
100
10
counts / min^2
5
10
15
20
25
30
35
40
45
50
191 k (x)
Minutes

of three such experiments. In spite of the short incubation period high K was still able to produce some increase in Ca^{45} efflux, which appears to indicate that not all Ca in the fraction affected by K depolarization is slowly exchanging.

The above evidence thus indicates that high K depolarization liberates some superficial Ca, part of which exchanges very slowly and which is in parallel with the remainder of the cellular exchangeable Ca.

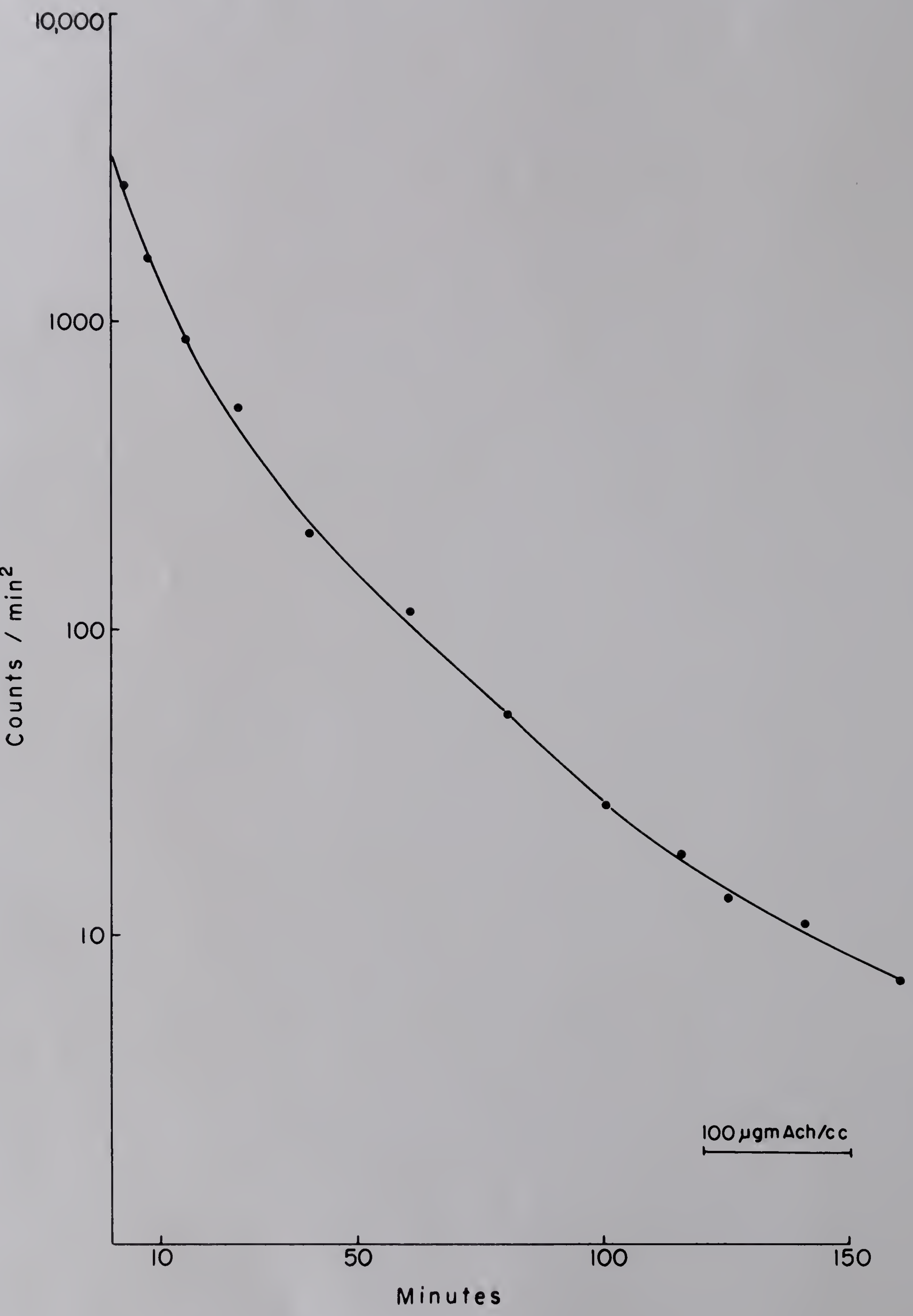
The effect of acetylcholine on Ca^{45} efflux.

The fact that high K depolarization releases some Ca from the cell surfaces suggested that this Ca might be involved in stabilizing the cell membranes and that its release might accompany increased membrane permeability and depolarization. If this superficial Ca fraction had such a specific function, it would be expected that acetylcholine, which increases membrane permeability and causes depolarization, would also liberate Ca from this fraction.

This theory was tested by adding acetylcholine to the media late during the efflux period. The tissues were loaded for the usual 2 to 3 hours in Ca^{45} labelled K.R. The effects on efflux of concentrations of acetylcholine of 3, 10, and 100 ugm/ml were so tested. None of these concentrations had any effect as illustrated in Fig. 21 for 100 ugm acetylcholine/ml.

Although acetylcholine did not release Ca^{45} into the efflux

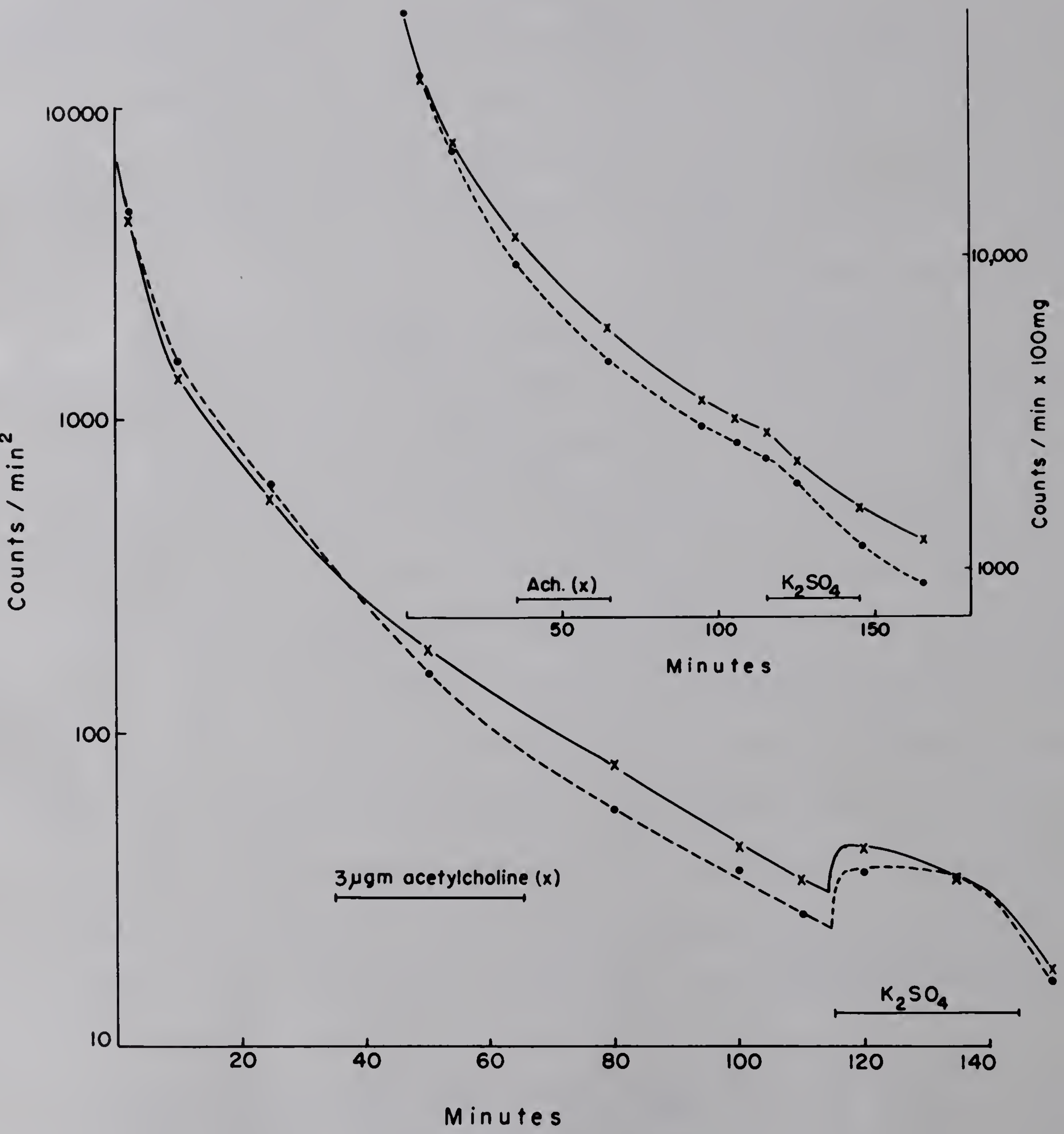
Fig 20. Rate of efflux of Ca^{45} from rat uteri which were loaded in Ca^{45} labelled K.R. for ten minutes. Efflux medium was changed from K.R. to 191 K for the period indicated by the horizontal bar in case of the experimental uteri (X). Each point is an average of 3 determinations.


10,000
1000
100
10
Counts / min²
10
50
100
150
Minutes
100 μgm Ach/cc

media, it was still possible that a release from a superficial fraction occurred, but that the released Ca^{45} entered the cells. Since the Ca in the superficial fraction is in parallel with the intracellular Ca this possibility could be tested for by testing the effect of early acetylcholine exposure on the high K induced increased Ca^{45} efflux at a later time. Fig. 22 gives the average results of four such experiments. Even a 40 minute exposure to acetylcholine did not release Ca^{45} from the superficial fraction. The rate of efflux from the acetylcholine treated horns is seen to be slightly higher than that from the controls. This could be due to higher Ca^{45} content of the experimental horns as shown in the inset of Fig. 21.

The failure of acetylcholine to release the Ca which is released by high K concentrations disproves the hypothesis that this superficial Ca fraction is necessarily associated with depolarization and increased membrane permeability. Indeed it seems more likely that the high K concentration merely displaces superficial Ca by an ion exchange effect, and these experiments failed to demonstrate a physiological function for this Ca. It must be emphasized that these results do not disprove the theory that acetylcholine causes its effects by displacing Ca from the membranes. If such stabilizing membrane Ca were small in total concentration and exchanged as fast or faster than the intracellular Ca its presence could not have been demonstrated in the experiments described here. The effect of acetylcholine on the Ca^{45}

Fig. 21. Rate of efflux from a uterine horn loaded in Ca^{45} labelled K.R. for two hours. At the time indicated 100 µgm acetylcholine/cc K.R. was added.



10,000
1000
Counts / min x 100mg
Ach. (x)
K₂SO₄
50
100
150
Minutes
10000
1000
100
10
Counts / min²
3μgm acetylcholine (x)
K₂SO₄
20
40
60
80
100
120
140
Minutes

efflux in two modified media were also tested. Fig. 23 shows that acetylcholine does not affect the loss of Ca^{45} into 191 K. As will be shown later this is contrary to its effect on influx from 191 K in which case it appears to increase the permeability for Ca moving inwards.

Fig. 24 shows that acetylcholine also does not affect the efflux of Ca^{45} into Ca free K.R. This finding does not support the hypothesis proposed by Edman and Schild, (12) which states that acetylcholine increases the rate of loss of contractility in Ca free media by increasing the rate of loss of Ca from the tissues.

Effects of high potassium depolarization and acetylcholine on Ca^{45} and total Ca uptake.

It is a widely held view that contraction of smooth muscle is initiated by an increased Ca influx resulting in a net increase in tissue Ca (13 - 16). Other investigators have obtained evidence that the involvement of Ca is more complex and that the release of bound Ca is also necessary for contraction (5, 12, 17 - 19).

The most direct way of testing the theory that contraction is

Fig. 22. Uterine horns loaded with Ca^{45} for 2 hours before efflux into K.R. and 191 K. All uterine horns were exposed to 191 K for the time indicated and the experimental horns (X) were also exposed to 3 μgm acetylcholine/ml K.R. during time indicated by the first horizontal bar. The upper curves show the loss of Ca^{45} and the lower curves show the rate of loss of Ca^{45}. The experimental and control horns came from the same rats. Each point is the average of 4 determinations.

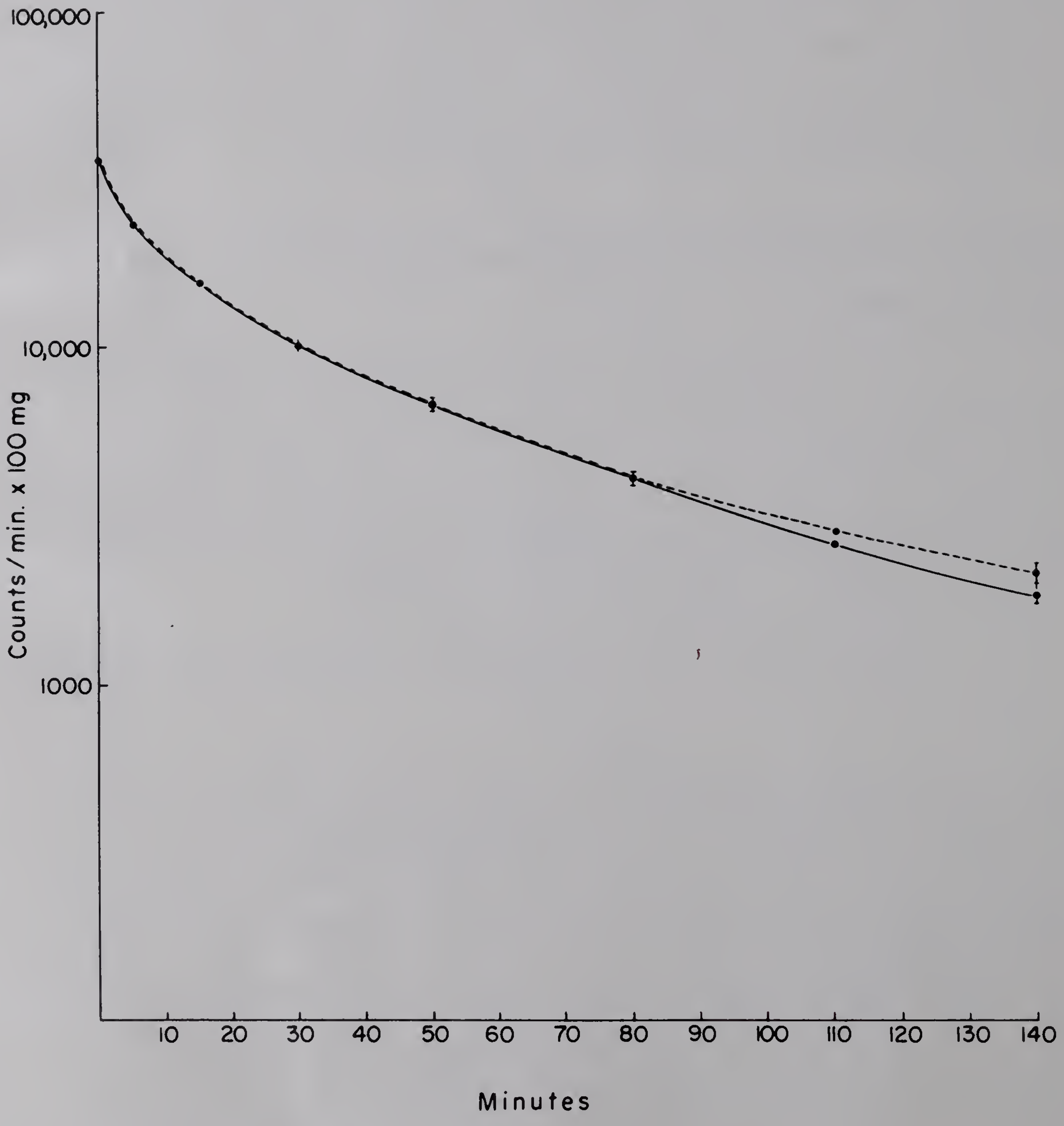
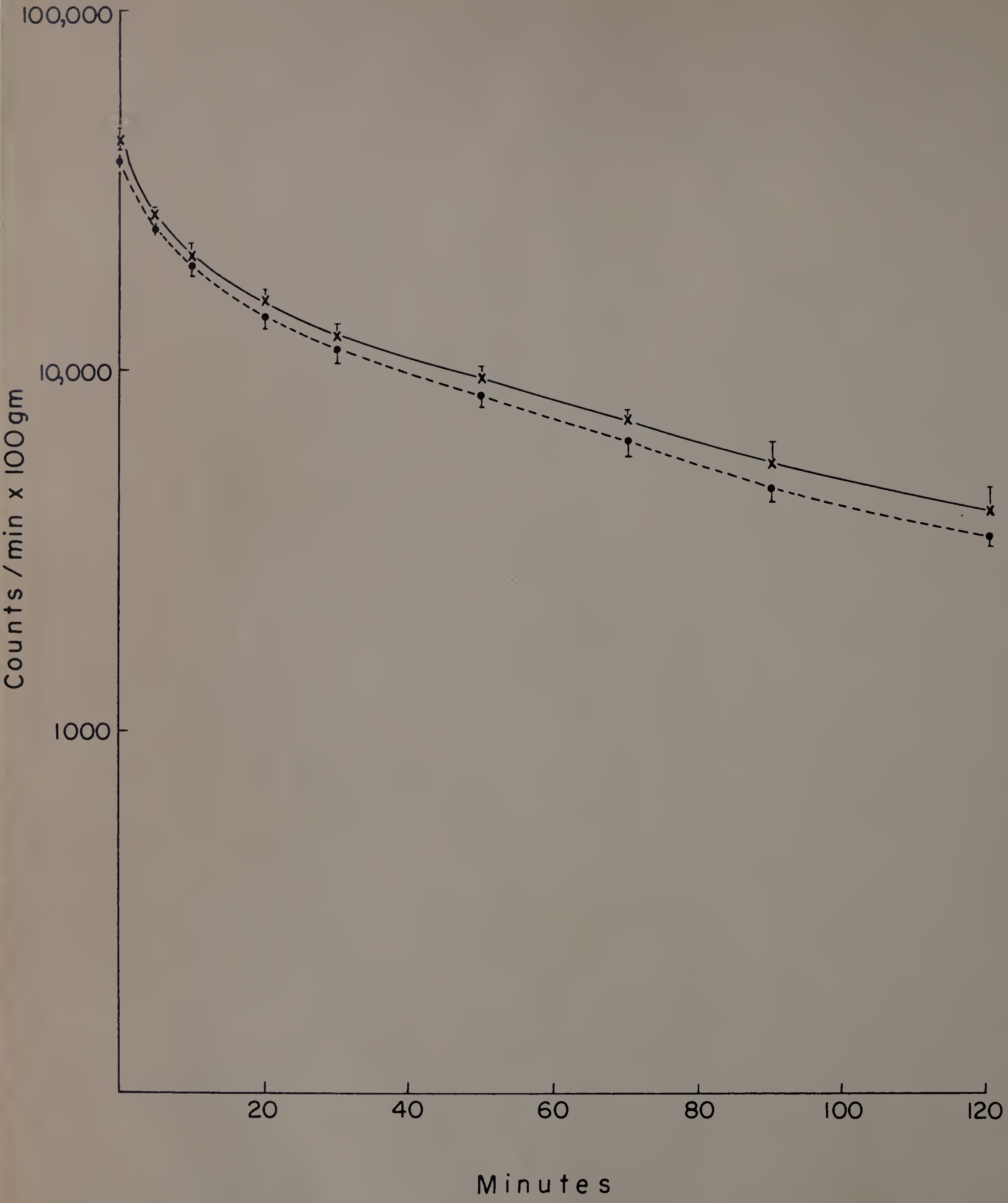

100,000
10,000
1000
Counts / min. x 100 mg
10
20
30
40
50
60
70
80
90
100
110
120
130
140
Minutes

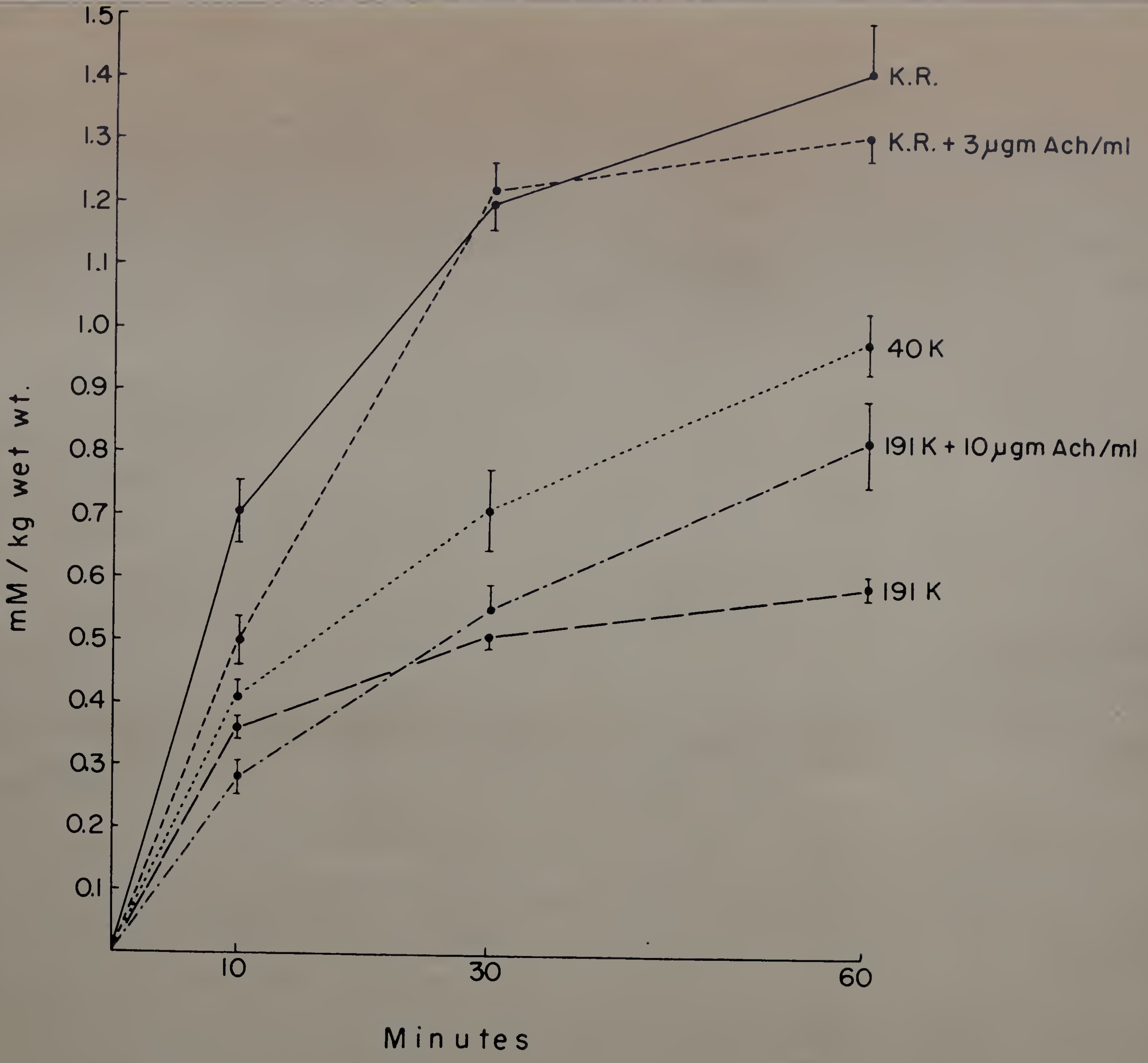
Fig. 23. Efflux of Ca^{45} into 191 K containing 10 μgm acetylcholine/ml (dashed curve) and into 191 K (solid curve). The two curves are from opposite uterine horns from the same rats. Each point is an average of 5 determinations. Twice the S.E. is indicated by the length of the vertical bars. Where bars are absent 2 times S.E. is less than diameter of the dots.


100,000
10,000
1000
Counts / min x 100 gm
20
40
60
80
100
120
Minutes

induced by a net Ca influx, is to measure the influx of Ca^{45} and net Ca movement during a contraction. Fig. 25 compares the influx of labelled Ca during four different conditions causing contractions with the control influx. At time zero the uteri were placed in the labelled solution which is indicated beside each curve and were removed and analyzed after 10, 30, and 60 minutes. In all solutions except the control K.R. the uteri were observed to be contracted throughout the hour of incubation. Nevertheless only the uteri in K.R. containing acetylcholine has an uptake similar to the controls and those in the high K depolarizing solutions had a markedly slower influx of labelled Ca than the controls.

The decrease of Ca^{45} uptake during the first ten minutes in the contracting uteri can best be explained by the fact that contraction of smooth muscle can greatly diminish the extra-cellular space (20). This explanation is also in accordance with the observation from Fig. 25 that addition of acetylcholine seems to further diminish the initial Ca^{45} uptake, since acetylcholine has been shown to potentiate a high K contraction. The observation that the initial uptake from acetylcholine containing K.R. is larger than that from the high K solutions can also be explained by considering the effect of contraction on the extracellular space. At 37 degrees C high K induces a contracture which is main-

Fig. 24. Efflux of Ca^{45} into Ca free K.R. (solid curve) and into Ca free K.R. containing 3 μgm acetylcholine/ml (dashed curve). The two curves are from opposite horns of the same rats. Each point is the average of 5 determinations. Bars on one side of points equal S.E. Absence of bars means that S.E. is less than diameter of points.


1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
mM / kg wet wt.
10
30
60
Minutes
K.R.
K.R. + 3 μgm Ach/ml
40 K
191 K + 10 μgm Ach/ml
191 K

tained at a perfectly steady level for as long as the K concentration remained elevated (21). This means that a mixing effect of the contraction so induced is likely to be of little importance, since the extracellular fluid is merely initially squeezed out. On the other hand acetylcholine increases the frequency of action potentials and the contraction is irregular, so that the mixing effect in the extracellular space would be considerable thus speeding up the uptake of Ca^{45}.

Durbin and Jenkinson (13) showed that acetylcholine causes an increase in non-specific membrane permeability in depolarized smooth muscle of the guinea pig taenia coli and causes a small increase in Ca^{45} uptake during 7 minutes exposure to Ca^{45} high K depolarizing solution. Comparison of the curves for 191 K and 191 K acetylcholine in Fig. 25 also shows such an acetylcholine induced Ca^{45} uptake in the rat uterus. Since the rate of Ca^{45} uptake becomes faster in the acetylcholine treated depolarized uteri only after 10 minutes (the time required for exchange of extracellular Ca) and since the site of acetylcholine action is believed to be at the cell membrane (13), this acetylcholine induced increased Ca uptake is taken to be an increased rate of Ca entry into the cells.

Finally Fig. 25 demonstrates that the amount of decrease of

Fig. 25. Ca^{45} uptake by rat uterine horns from different Ca^{45} labelled media (indicated by each uptake curve). Points of 3 lower curves are averages of 5 separate determinations and of two upper curves of 4 separate determinations. Vertical bars equal twice S.E. Bars projecting from one side of point equal S.E.

Ca influx is related to the degree of depolarization. Casteels and Kuriyama report (22) that at elevated external K concentrations a ten fold increase in K concentration causes a 32 mV change in the membrane potential of the non pregnant rat uterus. According to this figure and taking the internal K concentration as 181 mMolar (8), an external K concentration of 40 mMolar depolarizes the membrane to about -21 mV (inside minus out), and an external K concentration of 191 mMolar completely abolishes or slightly reverses the membrane potential. Thus as the inward electrical driving force for Ca ions is diminished to slightly less than one half the Ca influx is markedly reduced, and when the driving force is abolished the influx of Ca is reduced even further. These results could be most easily explained by assuming that, at least during one phase of membrane permeation, Ca penetrates the cell membranes in ionic form or as part of a positively charged complex.

Although these results very strongly suggest that high potassium induced contractions are not accompanied by a net Ca influx in the uterine smooth muscle more complete evidence is necessary to prove this point. Such evidence was obtained for the depolarization contraction induced by 191 mMolar K. For a net influx to take place during this procedure it can be calculated from data in Fig. 25 that the efflux would have to decrease at least to one third of its control value. However fig. 17 shows that the first depolarization had no discernable effect on the Ca^{45} efflux. This suggests that the high K depolarization was accompanied by a net Ca efflux instead of an increased net influx. This was verified by measuring the effects of 191 K on the total Ca concentration and on the total exchangeable Ca concentration.

For the first determination uterine horns were simply left for two hours in 191 K, while other horns serving as controls were left for two hours in K.R. The depolarized uteri had an average total Ca concentration of 1.8 ± 0.08 mM/kg wet wt. (4) and the controls 2.2 ± 0.09 mM/kg wet wt. (4) The effect of 191 K on the total exchangeable was determined by allowing the exchangeable Ca to become labelled by incubating the uterine horns for 140 minutes in Ca^{45} labelled K.R. and then transferring them to 191 K, labelled with Ca^{45} at the same specific activity as the labelled K.R., for one hour. The controls were left in labelled K.R. for 200 minutes. The total amount of exchangeable Ca in the depolarized horns was 1.12 ± 0.10 mM/kg wet wt. (4), and in the controls was 1.67 ± 0.047 mM/kg wet wt. (4). In both measurements the depolarized uteri had significantly less total Ca or Ca^{45} than the controls ($P < 0.01$). Thus the high K induced contraction was accompanied by a net efflux of about 0.5 mM/kg wet wt. This is not due to a loss of ionic extracellular Ca by a reduced extracellular space during contraction since the exchangeable cellular Ca concentration is about 2.9 mM/liter of cell water and the extracellular Ca concentration is 1.5 mMolar. Thus loss of extracellular fluid would slightly raise the total exchangeable tissue calcium expressed per unit weight.

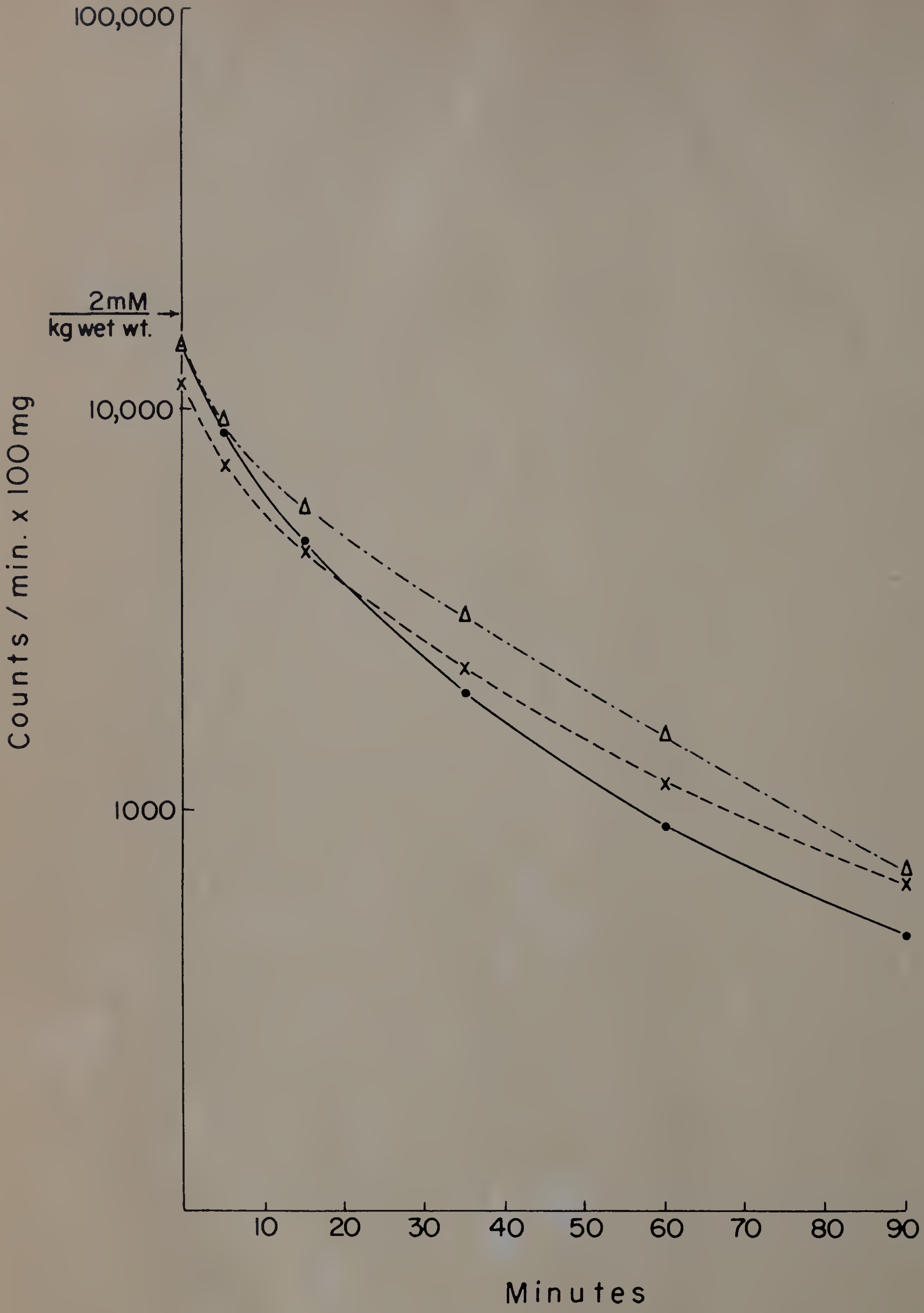
If for convenience the approximating assumption is made that after the first ten minutes the influx illustrated in Fig. 25 is due to penetration of Ca^{45} into the cells then the Ca influx into the cells during one hour is 0.47 mM/kg wet wt. greater in the uteri incubated in K.R. than in those incubated in 191 K. Thus the decreased Ca uptake in 191 K accounts almost entirely for the net Ca loss induced by this procedure.

The above results prove that the contraction induced by 191 mMolar K is not caused by a net inward movement of Ca*. They are also evidence against such a hypothesis for contractions induced by other K concentrations and acetylcholine.

The results presented in Fig. 25 suffer from the fact that the change in extracellular space and the mixing effect of periodic contractions prevented a direct comparison between the Ca uptake during contraction and rest. In order to make a direct comparison possible the following experimental procedures were carried out. Uterine horns were mounted as for the efflux experiments, and incubated for 10 minutes in Ca^{45} labelled K.R. They were then transferred to a tube containing the Ca^{45} labelled experimental test solution for a time T, following which they were washed in 5 tubes containing Ca^{45} labelled K.R. for a total time of 10 minutes. The tissues were then effluxed for an appropriate length of time, blotted, weighed, ashed and analyzed for Ca^{45}. The control uteri were treated exactly the same way except that the test solution in this case was Ca^{45} labelled K.R. The specific activity of the labelled test solutions and labelled K.R. were made to be the same so that the tissue counts could be compared directly. The initial 10 minutes in labelled K.R. served to equilibrate the

---

* Although the decrease in influx brought about by 191 K was too great to have been produced by even an absence of influx in the endometrium only, a check was done with carefully dissected longitudinal muscle. In an average of three experiments the labelled Ca gain between 10 and 30 minutes after the onset of Ca^{45} influx was 0.33 mM/kg wet wt. in the controls but only 0.14 mM/kg wet wt. in the depolarized uteri.

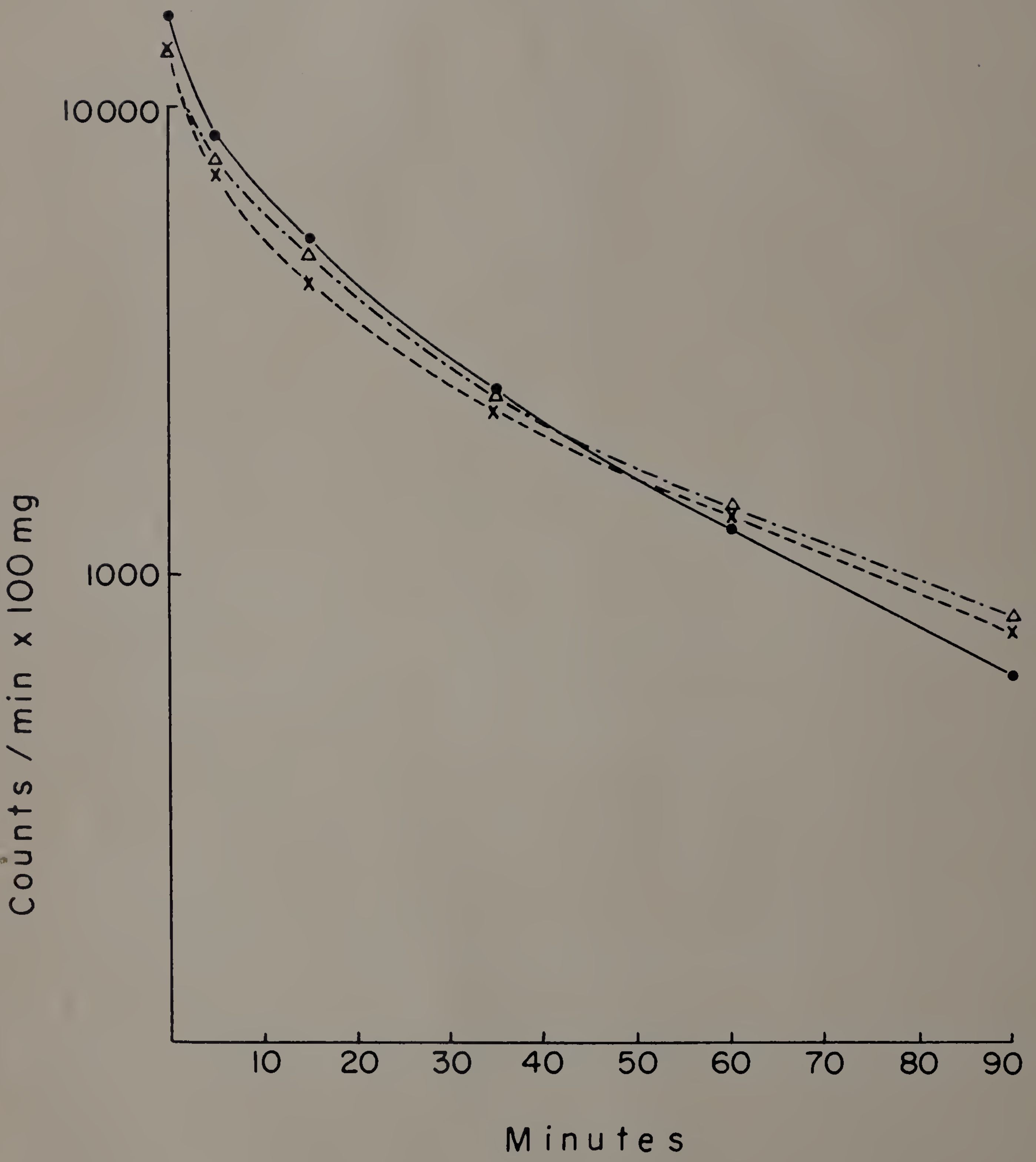


100,000
2mM
kg wet wt.
10,000
1000
Counts / min. x 100 mg
10
20
30
40
50
60
70
80
90
Minutes

extracellular space, and the final 10 minutes of washing in labelled K.R. served to relax the contracted uteri and thus restore the ratio between extra and intracellular spaces.

When the test solution was K.R. containing 3 μgm acetylcholine/ml, and T was 10 minutes the uptake expressed in mM/kg wet wt. was 1.32 ± 0.05 (5) for the experimental and 1.33 ± 0.048 (5) in the control uteri. Fig. 26 shows the results obtained when the test solutions were 191 K and 191 K containing 10 gm acetylcholine, and time T was 30 minutes. It is evident that although 191 mMolar K induced an observable contraction, the uptake of Ca^{45} was less than in the controls. The addition of acetylcholine to the depolarizing solution increased the Ca influx, bringing it to a value which equals the control uptake. However Fig. 26 demonstrates what could be an important phenomenon of Ca exchange during contraction, namely the Ca^{45} which was taken up during contraction is released more slowly than the Ca^{45} taken up by the tissues at rest. This can be seen by the crossing of the 191 K curve above the control curve after 20 minutes, and by the fact that after 10 minutes the control efflux curve falls below the 191 K plus acetylcholine efflux curve. These results could be explained by stating that contraction is not necessarily dependent

Fig. 26. Rat uterine horns were incubated in Ca^{45} labelled K.R. for 10 minutes and then for a further 30 minutes in Ca^{45} labelled K.R. (·), in Ca^{45} labelled 191 K (X), Ca^{45} labelled 191 K containing 10 μgm acetylcholine/ml (△). The horns were then washed in Ca^{45} labelled K.R. for another ten minutes and effluxed into K.R. for 90 minutes. Each point is the average of 5 determinations.


10000
1000
Counts / min x 100 mg
10
20
30
40
50
60
70
80
90
Minutes

upon a movement of Ca from the outside into the cells, but is accompanied or preceded by a movement of Ca from a more readily exchangeable cellular fraction into a more slowly exchangeable fraction. The meaning of such a possible Ca movement accompanying contraction will be explored in the discussion.

Fig. 27 shows the same type of experiment using the test solutions K.R. containing 3 μgm acetylcholine/ml and 191 K containing 10 μgm acetylcholine/ml. The same conclusions as before may be drawn. The contraction during influx did not increase the Ca^{45} uptake, but did slow down the efflux of Ca^{45} taken up during the experiment.

To test the possibility that the lower efflux rate following Ca^{45} loading during contraction was caused by an uptake in the inexchangeable fraction, the efflux curves were compared after loading in a Ca^{45} labelled K.R. containing 3 μgm acetylcholine/ml for 3 hours on the one hand and loading for three hours in labelled K.R. on the other hand. Fig. 28 shows no difference between the two procedures showing that the inexchangeable Ca is not involved.

Fig. 27. Rat uterine horns were incubated in Ca^{45} labelled K.R. for 10 minutes and then for 30 minutes in Ca^{45} labelled K.R. (· 4 horns), in Ca^{45} labelled 191 K containing 10 μgm acetylcholine/ml (△ 3 horns), and in Ca^{45} labelled K.R. containing 3 μgm acetylcholine/ml (X 4 horns). The horns were then washed in Ca^{45} labelled K.R. for another ten minutes and effluxed into K.R. for 90 minutes.

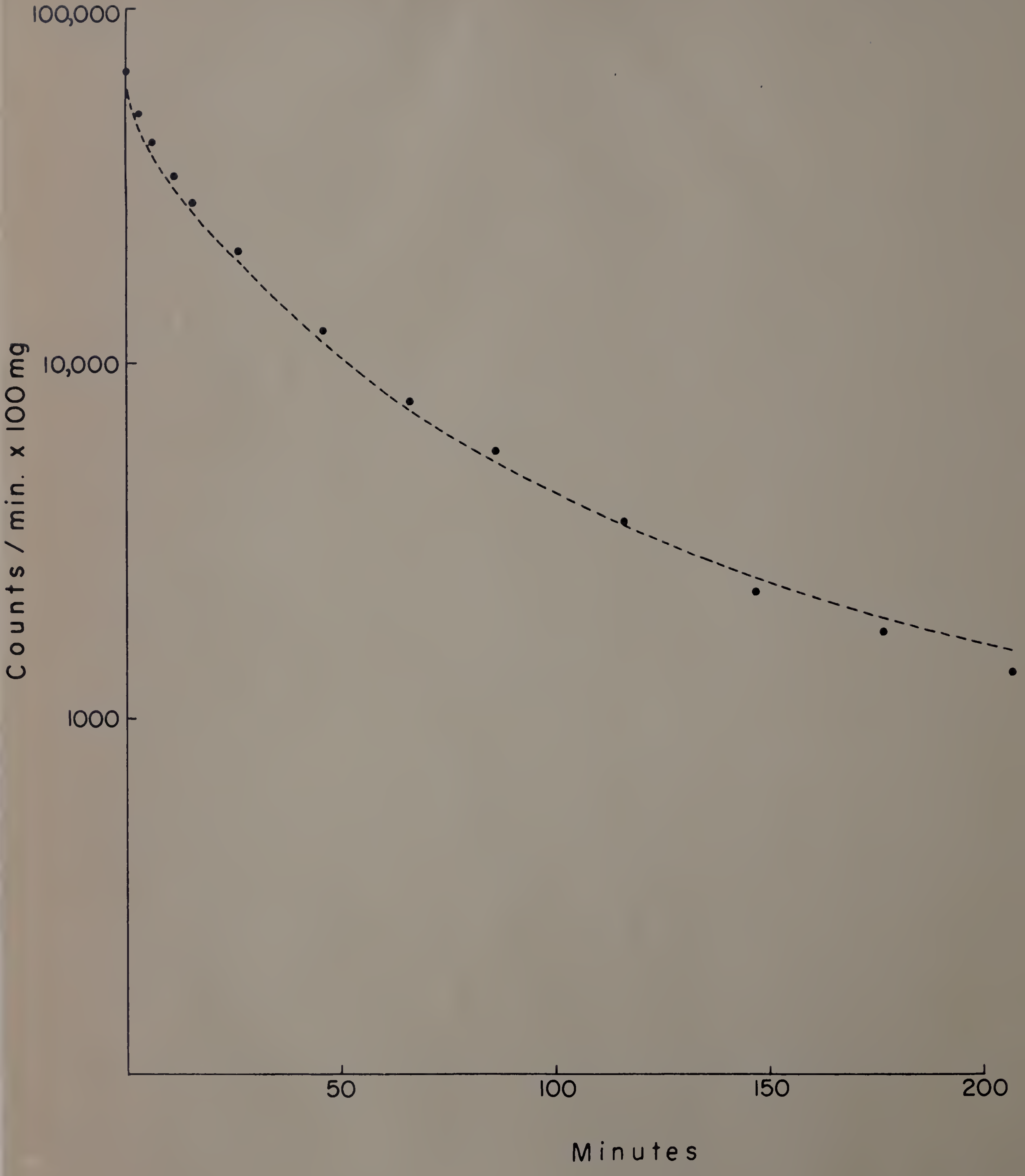

100,000
10,000
1000
Counts / min. x 100 mg
50
100
150
200
Minutes

Fig. 28. Rat uterine horns were incubated in Ca^{45} labelled K.R. for 3 hours, and opposite horns from the same rats were incubated in Ca^{45} labelled K.R. containing 3 μgm acetylcholine/ml for the same time. The horns were then effluxed into K.R. for 210 minutes. Ca^{45} per 100 mg of tissue during efflux is given by the dots for the acetylcholine treated horns and by the dashed curve for the control uterine horns. Each efflux curve is the average of 5 separate efflux experiments.

EFFECTS OF Ca FREE MEDIA ON Ca^{45} EFFLUX

METHODS

Solutions.

The following substitutions were made as indicated in results: Equimolar amounts of $SrCl_2$ and $BaCl_2$ for $CaCl_2$ in K.R. In presence of Ba the K.R. was made SO_4 free by substitution with Cl. Equimolar amounts of CholineCl were substituted for NaCl in K.R.

Procedures.

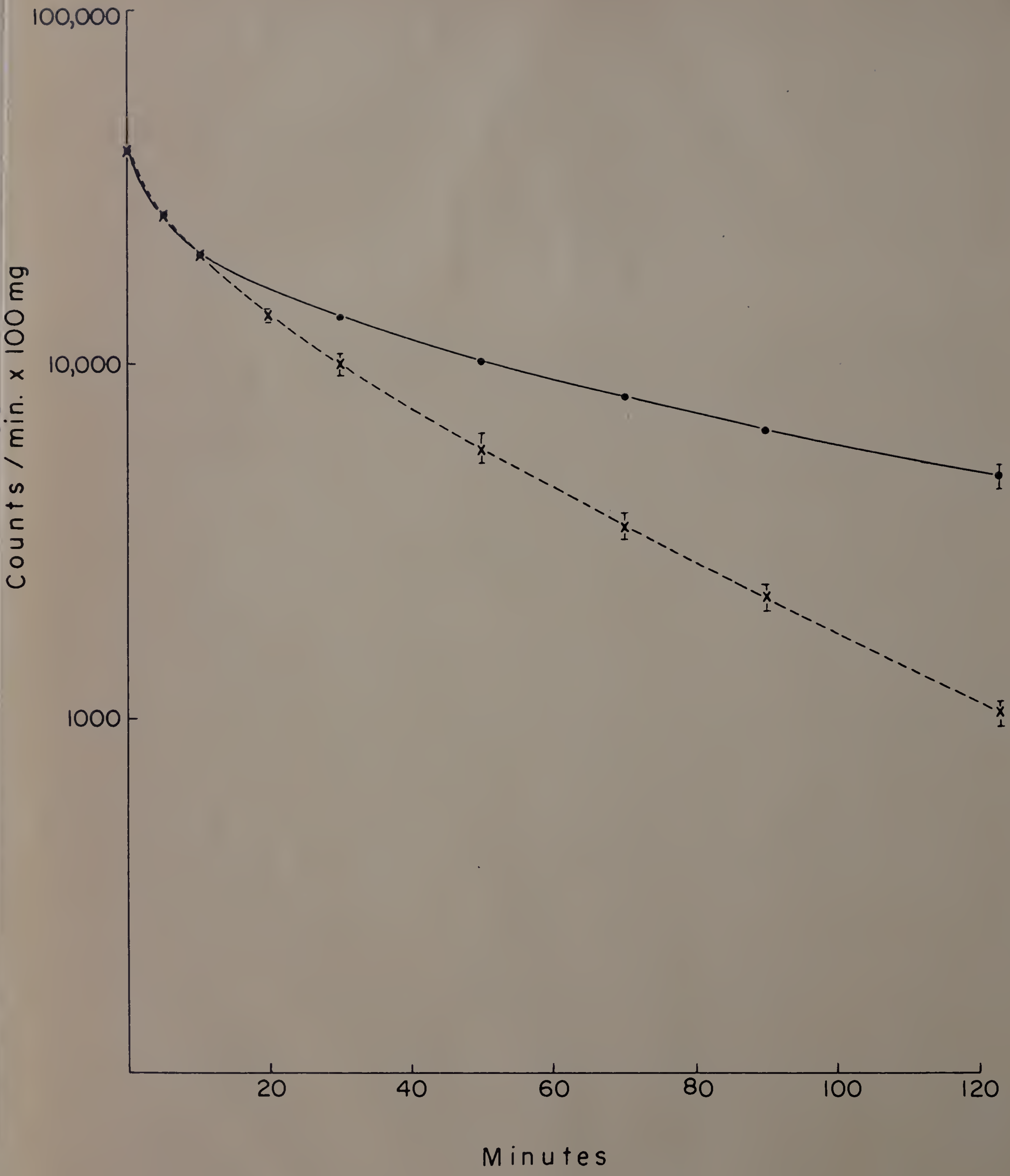
Generally the same as before. Details are given in results.

RESULTS

These experiments were performed in the hope that they would provide further information about the Ca fraction involved in contraction and would also shed some light on the exchange of calcium across the cell membrane.

Investigations by Edman and Schild (12) and Daniel (15) demonstrated that uterine contractile response to acetylcholine disappeared slowly in Ca free solutions. However the addition of EDTA to the Ca free medium caused a rapid disappearance of the contractile effect (15). If the action of EDTA was a more rapid removal of the Ca fraction involved in the initiation of contraction a marked increase in Ca^{45} efflux would be anticipated.

Since there is convincing evidence that Ca becomes bound to the lipid or lipoprotein components of cell membranes (23 - 26) it is of interest to know if passage of Ca through the membrane is dependent on the presence of extracellular Ca.

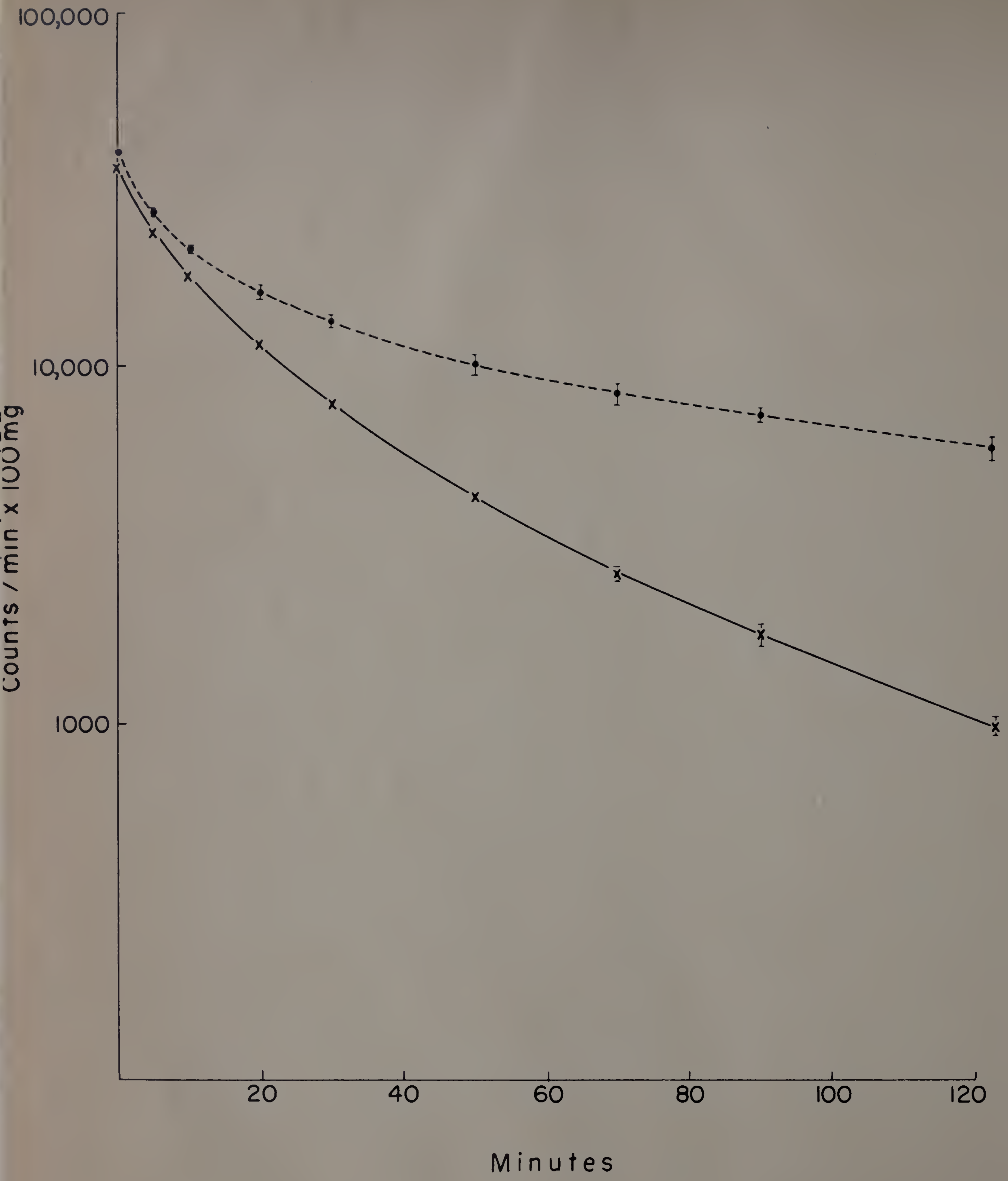

100,000
10,000
1000
Counts / min. x 100 mg
20
40
60
80
100
120
Minutes

Effects of absence of Ca and EDTA on Ca^{45} efflux.

Figures 29 and 30 compare respectively the efflux into Ca free K.R. with efflux into K.R., and the efflux into Ca free K.R. containing 2 mMolar EDTA, with a control efflux of Ca^{45} into K.R. (Ca free) The uteri were placed in the different solutions immediately following rinsing after loading with Ca^{45}.

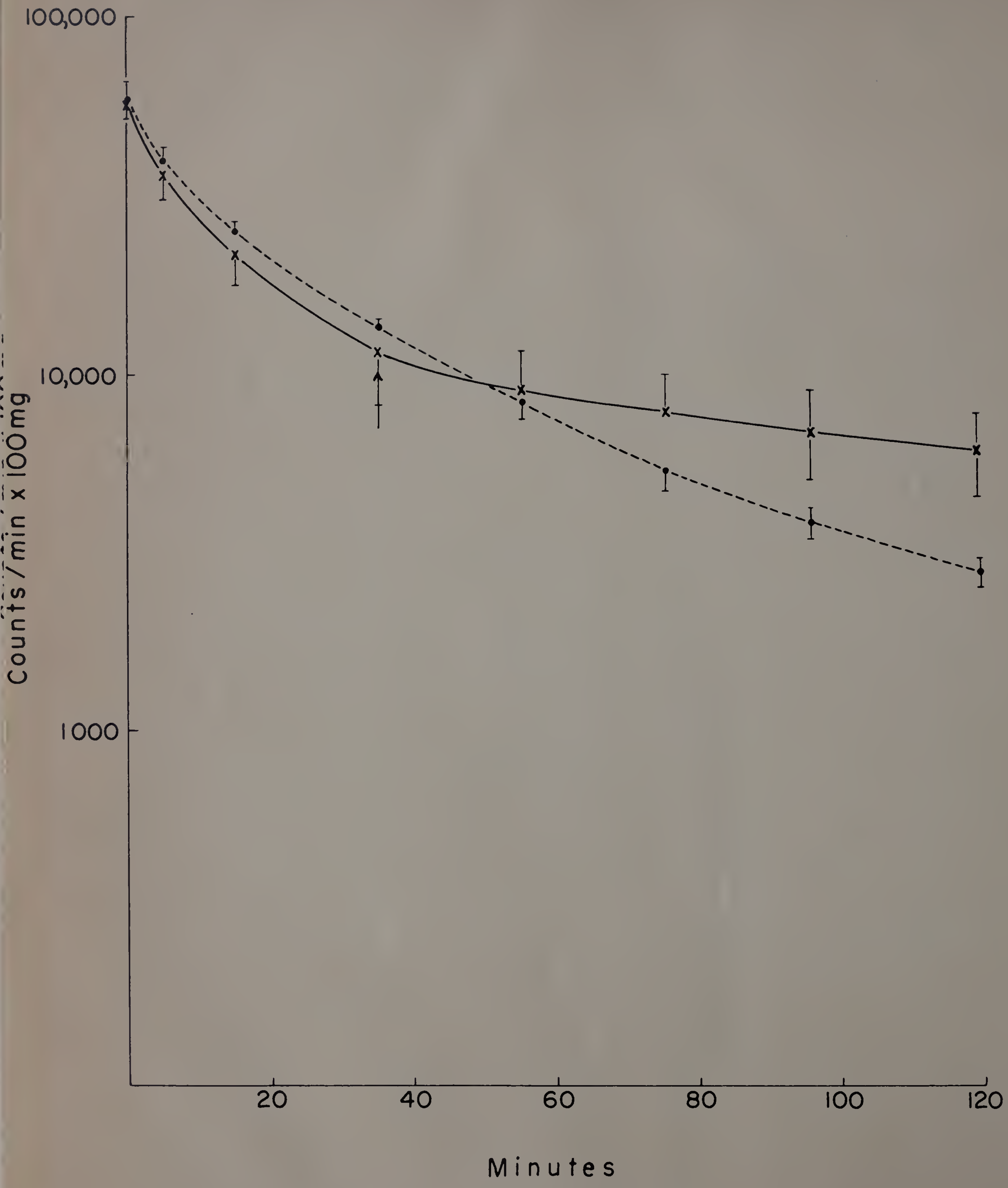
No change in efflux is seen during the first 10 minutes of efflux indicating that the efflux of extracellular Ca^{45} is not noticeably effected. However the efflux of cellular Ca^{45} is slowed down very markedly by the omission of Ca from the incubation medium. The addition of EDTA to Ca free medium reversed this effect. These experiments were done on the same batch of rats and all loading solutions had identical Ca^{45} specific activities making possible direct comparison of the curves in figures 29 and 30. Such a comparison shows that a Ca free solution containing EDTA increases the Ca^{45} efflux over the control efflux into K.R. for the first hour (except for first 10 minutes). This leads to a greater curvature of the Ca^{45} efflux curve in presence of EDTA. The effect of EDTA is fully reversible upon its removal from the Ca free solution as may be seen in Fig. 31. Thus the effect of EDTA to increase efflux requires its continued presence and is reversed on its removal even though Ca was not supplied to the tissue. This suggests that unstabilization of the membrane by Ca depletion is not the mech-

Fig. 29. Ca^{45} efflux into K.R. (X), and into Ca free K.R. (·). The two procedures were done on opposite uterine horns from the same 5 rats. Vertical bars indicate twice the S.E. In absence of bars twice the S.E. is less than the diameter of the points.



100,000
10,000
1000
Counts / min x 100mg
20
40
60
80
100
120
Minutes

Fig. 30. Ca^{45} efflux into Ca free K.R. (·), and into Ca free K.R. containing 2 mMolar EDTA (X). The two procedures were done on opposite uterine horns from the same 5 rats. Vertical bars indicate twice the S.E. In absence of bars twice the S.E. is less than the diameter of the points.

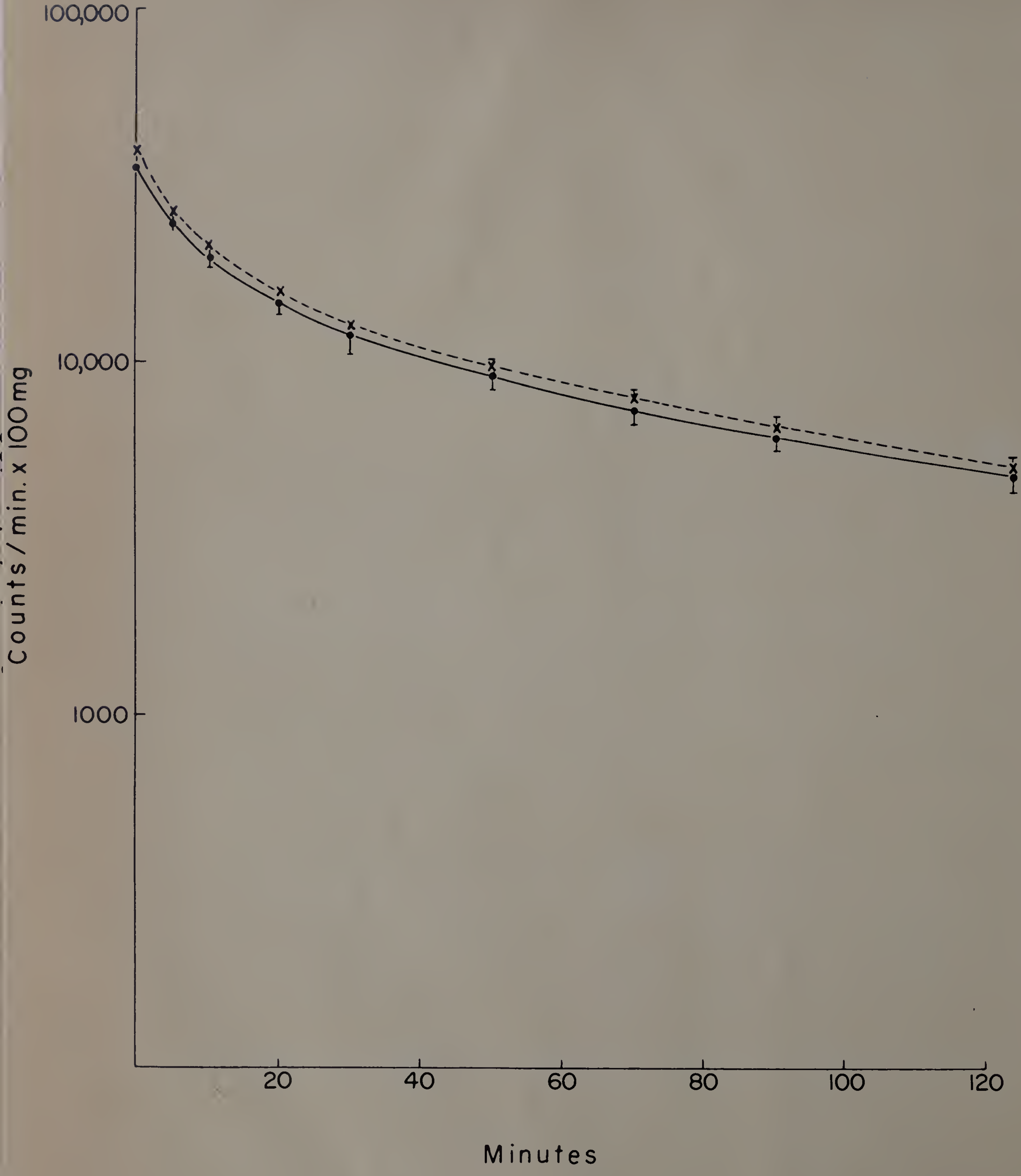

100,000
10,000
1000
Counts/min x 100mg
20
40
60
80
100
120
Minutes

anism of the EDTA effect. EDTA does not increase the Ca^{45} efflux by removal of Mg since efflux into a Ca free, Mg free solution did not differ from efflux into Ca free solution (see Fig. 32). In the previous section it was also shown that another Ca chelating agent EGTA at a concentration too low to significantly lower the Mg concentration increased the Ca^{45} efflux above the efflux into K.R. (see Fig. 19).

In order to clarify the remaining part of this section it is necessary to postulate a rather general hypothesis explaining the above results. Phospholipids extracted from cell membranes (26,27), isolated eythrocyte membranes (23,27), and isolated skeletal muscle membranes (25) all show the presence of sites which associate with Ca. Mikulecky and Tobias (27) presented evidence that these sites do not merely require the presence of Ca as counter-ions to neutralize fixed negative charges, but form "weak salts" with Ca. Thus without at present going into the actual mechanism whereby Ca ions move through the membrane, two very probable steps in cellular Ca exchange are the association of Ca with and it's dissociation from negatively charged sites in the membrane.

Before Ca^{45} can leave the cells when Ca efflux is being measured, it has to be released from sites in the membrane. It is

Fig. 31. Ca^{45} efflux into Ca free K.R. containing 2 mMolar EDTA (.), and into same for 35 minutes followed by Ca free K.R. for the remainder of the efflux period (X). The two procedures were done on opposite uterine horns from the same 4 rats. Vertical bars indicate twice the S.E. Bars projecting from one side of the points indicate S.E.



100,000
10,000
1000
Counts / min. x 100 mg
20
40
60
80
100
120
Minutes

postulated that this release of labelled Ca from the membrane is enhanced by the presence of unlabelled Ca or by the chelating action of EDTA or EGTA, and the enhanced release is observed as an increased Ca^{45} efflux.

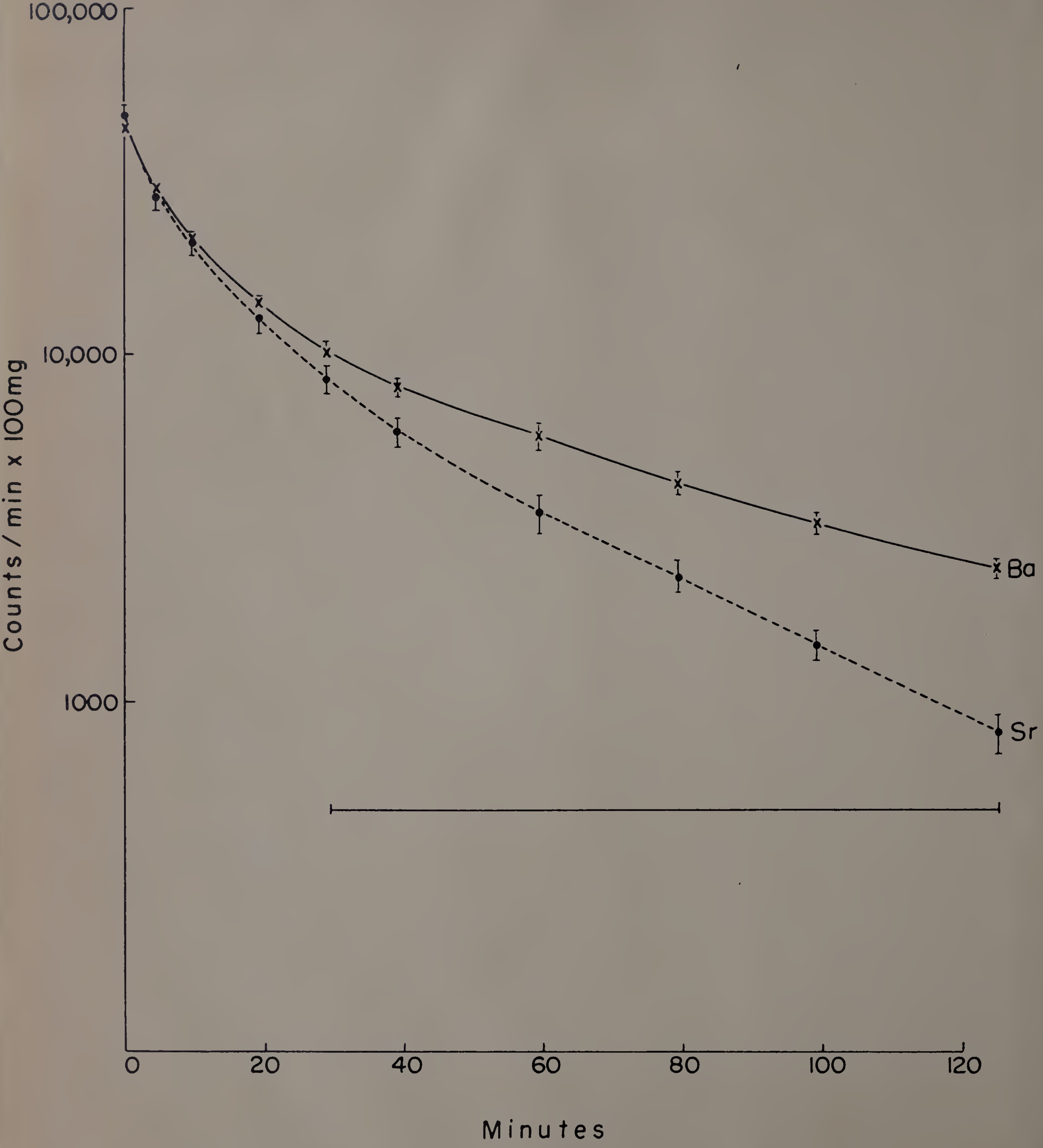
Effects of Sr, Ba, Mg and Na on Ca^{45} efflux into Ca free K.R.

The ability of extracellular Ca to release Ca from the membrane must be due to its affinity for the membrane sites. Thus by varying the ion content of the efflux solution it is possible to obtain information about the relative specificty of the membrane sites for various cations. It must be stated that there may be other negatively charged groups in the membrane which are not concerned with Ca exchange across the membrane. Such sites are of course not involved in this study.

Omission of the usual 1 mMolar Mg from the Ca free K.R. did not obviously affect the rate of Ca^{45} efflux, as is demonstrated in Fig. 32.

The experiment illustrated in Fig. 33 compares the effects of substitution of Ca with Sr and with Ba. It is evident that Sr can replace Ca without any noticeable effect on efflux, but that Ba causes a reduction in the rate of efflux when it is substituted for Ca 30 minutes after the beginning of the Ca^{45} efflux. Comparison between Figs. 33 and 34 is possible since the specific activities of the loading solutions were the same and the times at which the efflux medium was

Fig. 32. Ca^{45} efflux into Ca free K.R. (·) and into Ca free Mg free K.R. (X). The two procedures were done on opposite uterine horns from the same 5 rats. Vertical bars indicate the S.E.


100,000
10,000
1000
Counts / min x 100mg
0
20
40
60
80
100
120
Minutes
Ba
Sr

changed to Ca free K.R. (with necessary substitution) was also the same and since the experiments were performed only a few days apart. Such comparison shows that Ba does increase the efflux somewhat over the Ca free solution without substitutions, but can not prevent the decrease in efflux as completely as does Sr.

Fig. 34 demonstrates that substitution of choline for Na in the Ca free K.R. has no effect on the reduction in the rate of efflux. Thus in conclusion the above results indicate that the Ca binding sites in the membrane have equal affinities for Ca and Sr, lower affinity for Ba, but do not bind Mg or Na.

Fig. 33. Ca^{45} efflux into K.R. for 30 minutes followed by K.R. with Ba substituted for Ca (X), and K.R. with Sr substituted for Ca (·). The two procedures were done on opposite uterine horns from the same 4 rats. Vertical bars indicate twice the S.E. Bars projecting from one side of point indicate S.E.

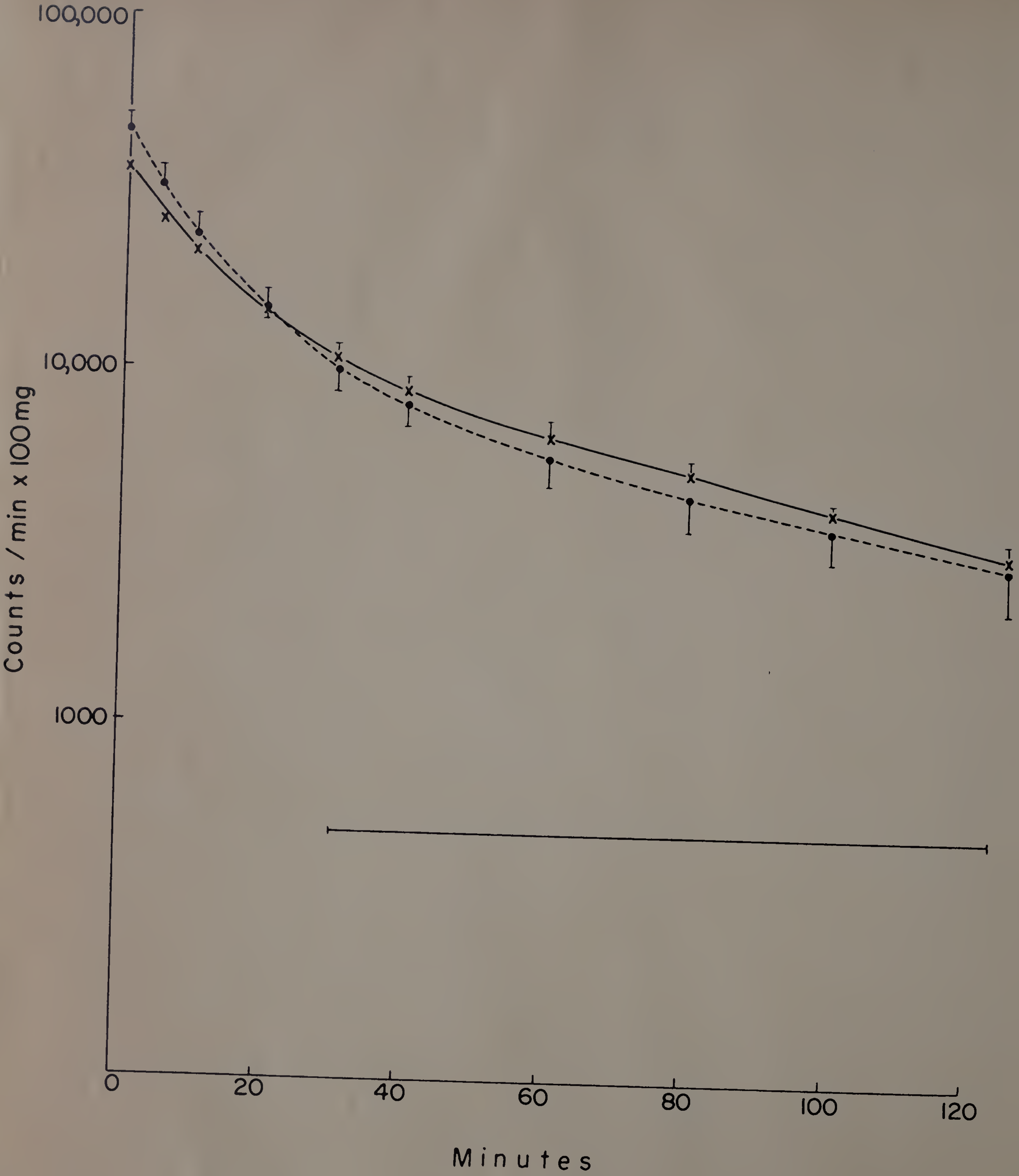

100,000
10,000
1000
Counts / min x 100 mg
0
20
40
60
80
100
120
Minutes

Fig. 34. Ca^{45} efflux into K.R. for 30 minutes, followed by Ca free K.R. (X), and by Ca free K.R. with CholineCl substituted for NaCl (·). The two procedures were done on opposite horns from the same 4 uterine horns. Bars projecting from one side of points indicate S.E. In absence of bars twice the S.E. is less than the diameter of the points.

DISCUSSION

Extracellular Uterine Calcium.

Since it has been an established fact for many years that plasma Ca is only partly ionized it is surprising that most in vitro bathing solutions contain Ca concentrations approximately equal to the total plasma Ca. The range of recently reported values for the percentage ultrafiltrable Ca in human serum is from 40 to 70% of the total serum Ca. (2). Munday and Mahy in a careful study in which temperature and pH were controlled, found a normal percentage of ultrafiltrable calcium of 52% of the total plasma Ca. This is in close agreement with the value of 53% found by means of equilibrium dialysis against pH buffered medium in this study on rat serum. Furthermore the absence of a rapid change in tissue Ca concentration, and the prolonged maintenance of the in vivo Ca concentration when rat uteri are incubated at an external Ca concentration of 1.5 mMolar (which is the dialyzable serum Ca concentration) strongly suggests that the interstitial ionic Ca concentration closely approximates the dialyzable serum Ca.

The knowledge of the extracellular ionic Ca concentration is not sufficient to determine the fraction of the total tissue Ca which is located extracellularly. The uncertainties arise from the facts that there is no undisputed method for measuring the extracellular fluid volume, and that an unknown quantity of Ca is bound to the connective tissue. Bohr (28) discusses methods for measuring the extracellular space and indicates that the inulin is the most acceptable and probably the most reliable method. By using the inulin space of 460 ml/kg wet wt. as reported by Daniel (4) for estrogen pretreated rat uteri and assuming equal activity coefficients for Ca in Krebs Ringer

bicarbonate solution, the calculated extracellular ionic Ca represents 0.69 mM/kg wet wt.

An approximate analysis based mainly on the initial rapid Ca exchange and the fact that a short exposure to EDTA could not increase the initial rate of Ca loss from the uteri led to an exchangeable extracellular Ca fraction of 0.8 mM/kg wet wt. The excess over the ionic Ca in the extracellular space is thus 0.11 mM/kg wet wt. or 0.24 mMoles/liter extracellular volume. This is in good agreement with the value of 0.2 mMoles loosely bound Ca per liter of extracellular fluid found by Bozler (7) in the frog stomach smooth muscle (the external Ca concentration in this case was 1 mMolar). In the same study Bozler found that fascia contains three times as much loosely bound Ca as does smooth muscle. In addition fascia has 1.32 m Mole inexchangeable Ca per kg tissue water. By comparing the smooth muscle and fascia Ca fractions Bozler reasoned that the connective tissue of smooth muscle would contain 0.44 mMoles inexchangeable Ca per liter extracellular fluid. If the rat uterus contained the same concentration of extracellular inexchangeable Ca, then this would account for an additional 0.2 mM extracellular Ca/kg wet tissue, or about half the inexchangeable tissue Ca (see below).

Bauer, Goodford and Hüter (14) also found evidence for extracellular Ca binding in the guinea pig taemia-coli in that their extracellular Ca space exceeded the inulin space. Evidence for extracellular Ca binding has also been presented for skeletal muscle (30, 31) and cardiac muscle (32, 33).

A recent study by Koketsu, Kitamura and Tanaka (25) shows that one needs to be cautious in directly comparing the Ca binding by collagen fibers in the extracellular space of muscle with that of

collagen in connective tissue. They isolated the sarcolemma of bull frog skeletal muscle. These isolated membranes had an outer layer of 1 - 2 μ thick composed of collagen fibers as well as the cytoplasmic membrane proper. After chloroform methanol extraction of lipoproteins and lipids the remaining collagen fibers were unable to bind radioactive calcium to a significant extent, but the surface film prepared from the extract did adsorb significant amounts of calcium. In any case, these findings would support the view that binding of exchangeable Ca in the extracellular space is very limited.

After discussing the extracellular Ca fraction it is logical to deal with the cellular Ca fraction. However since it was the main object of this thesis to investigate the properties of the cellular Ca fraction such a heading would be too broad. Instead the remainder of the discussion will be dealt with under a number of headings. However it should be pointed out that the division under these headings will be somewhat artificial since each part of the discussion will necessarily draw on almost all the results presented in this thesis.

The rate of rat uterine Ca exchange in K.R.

Since the total Ca concentration remained constant during the uptake and efflux of Ca^{45} the movements of tracer Ca were due to a true Ca exchange.

The observation that nearly all the exchangeable uterine cellular Ca becomes equilibrated with external Ca^{45} in one hour is in agreement with the general finding that Ca exchange across smooth muscle membranes ($t\ 1/2 = 2$ min. - 60 min.) (7, 14, 33-36) is more rapid than across cardiac muscle ($t\ 1/2 = 30 - 186$ min.) (31, 32) and skeletal

muscle sarcolemma (t 1/2 = 56 min. - 300 min.) (29, 30), and across squid axon neurolemma (t 1/2 = 20 hr) (38).

Rate constants for the Ca exchange of uterine cells could not be obtained, since the slopes of the plots of log Ca^{45} in the tissue vs. time for efflux and the log of the unexchanged fraction vs. time for influx decreased gradually with time. Furthermore, the slope of the curve relating the log of the counts emerging per unit time to time never became equal to the slope of the curve relating the log of the counts remaining in the tissue to time (Persoff 39). The non-exponential nature of the Ca exchange could result from many factors. Keynes (40) listed the following possible explanations for a similar exchange pattern for Na in frog skeletal muscle: (1) Binding in the extracellular space. (2) Trapping in some pockets of the extracellular space, which are less accessible than the remainder. These two factors would tend to flatten the initial part of the efflux curve. (3) Size distribution of the muscle cells. (4) Different membrane permeabilities for different types of cells. (5) Gain in total ion concentration during the efflux period. (6) A drop in the absolute size of efflux with time. (7) Intracellular binding. (8) Binding at the cell surfaces.

For Ca exchange in the rat uterus factors 5 and 6 are not likely to contribute significantly to the flattening of the efflux curves, since there was no gain of Ca during the exchange experiments. However a gradual decrease in both Ca influx and efflux was not ruled out. A possible reason for a much greater drop in the absolute size of the Na efflux in frog skeletal muscle than for such a drop if any in Ca efflux from rat uterus is the presence of active Na "extrusion" from skeletal muscle cells and of active Ca "exclusion" from uterine

smooth muscle cells. Thus if through time less metabolic energy became available for active Ca exclusion the influx of Ca would be increased, instead of a decrease of the efflux as would be the case in active extrusion.

In smooth muscle besides the size distribution another morphological factor would be the irregular shape of the smooth muscle cells with their relatively wide centers and tapering ends. The factors 1, 7 and 8 relating to ion binding are expected to be much more extensive for Ca than for Na. Harris (30) suggests the dissociation of Ca to be rate limiting in a statement which is very applicable to the results reported in this thesis: "There is no justification for attempting to interpret Ca movements in terms of a small number of separate first order processes because behaviour is variable and probably results from a continuous gradation in strength of binding of the element". Thus it is postulated that the inhomogeneity of the cellular Ca, which is exhibited in exchange with labelled Ca, is due mainly to the different rates of release of Ca from intracellular organelles, such as the mitochondria, nucleus, endoplasmic reticulum and the contractile actin and myosin fibrils and also to different rates of dissociation from different molecular groups in the organelles and cytoplasm.

Three pieces of evidence presented in the results provide support for the above nature of the inhomogeneity of cellular Ca. During the early part of Ca^{45} efflux from previously loaded uterine cells the above theory would predict that the Ca movement through the membrane would be more rate limiting than late in efflux, since at first the loosely bound Ca^{45} is involved. Thus at first the membrane phase of Ca exchange may to some extent obscure the gradation in strength of

intracellular Ca binding. It follows that any procedure which would accelerate the Ca movement through the membrane would accentuate the intracellular inhomogeneity. In the final section of the results it was shown that EDTA, which is believed to accelerate the membrane phase of Ca^{45} efflux (see discussion below) did indeed accentuate inhomogeneity by increasing the curvature of the semilogarithmic Ca^{45} efflux curve.

Secondly it was observed that contraction during loading with Ca^{45} slowed down the efflux of Ca^{45} from the relaxed uterus afterwards. This could again be readily explained on the basis of inhomogeneity of intracellular Ca by postulating that contraction was causing a shift of some more readily exchangeable Ca to more slowly exchanging Ca. Finally the Ca liberated by high potassium depolarization was clearly different from the bulk of cellular Ca.

The results presented in this thesis could be explained by assuming that the inhomogeneity of cellular Ca results mainly from different intracellular organelles and molecules binding Ca with different strengths.

Inexchangeable Ca.

In the rat uterus 0.45 mM/kg wet wt of Ca, or about 20% of the total tissue Ca was found to be inexchangeable or very slowly exchangeable. Such a Ca fraction has been found in all three types of muscle. In the skeletal muscle the inexchangeable Ca is about 40% of the total tissue Ca (40), and in cardiac muscle the inexchangeable Ca is about 30% (7,31,42). In smooth muscle values of 78% in guinea pig taenia-coli (35) and 30% in frog stomach musc.e (7) have been obtained for the

inexchangeable Ca fraction. However in a very recent publication Bauer, Goodford and Hüter (14) reported the absence of a significant inexchangeable Ca fraction. They attributed this to the use of a modified medium which contained no phosphate. Although this aspect has not been specifically investigated in this study the omission of phosphate from the bathing solution did not lower the total Ca concentration of the rat uterus. Schatzmann (43) found evidence which might be interpreted as support for the finding of Bauer et al. When he loaded with Ca^{45} the taenia-coli of the guinea pig in phosphate free medium and then effluxed it in a non-labelled medium of the same composition the efflux occurred at a much faster rate than the controls in phosphate containing solutions. This finding indicates that the slowly exchanging fraction was greatly reduced. However this does not give information about the Ca fraction which might not have become exchanged during the 140 minute loading period. [One possibility which may not have been excluded is that according to Langer (44) the presence of phosphates in the tissue ash may prevent complete titration of the Ca present with EDTA. Thus in the study of Bauer et al. it seems possible that the Ca determination by EDTA titration might have led to an underestimation of the total tissue Ca but not of the Ca in the phosphate free medium and consequently of the labelled tissue Ca concentration.]

Several sources have been suggested for the inexchangeable tissue Ca. As mentioned above Bozler attributes some inexchangeable Ca to the connective tissue. Brink (45) suggests that some protein bound Ca may be prevented from exchanging by virtue of phospholipid micelles surrounding the Ca protein complex. Hasselbach (41) states that Ca bound to actin fibrils and half of the Ca associated with myosin

fibrils is inexchangeable, and that some but not all of this Ca becomes exchangeable when the two proteins are combined. In all probability the inexchangeable Ca is also heterogenous in that a number of different sources contribute to this fraction.

Active membrane transport of Ca in rat uterus.

In the results it was shown that the cellular and extracellular Ca are not in electrochemical equilibrium with respect to each other, the intracellular Ca concentration being much lower than its equilibrium value. Such a Ca gradient would not require an active transport system if the cell membrane is impermeable to Ca ions. This possibility was excluded by demonstrating the exchange of cellular Ca with external Ca^{45}. However if the Ca exchange occurred only by an exchange diffusion mechanism, whereby a Ca ion passing outward through the membrane is necessarily coupled to the inward movement of another Ca ion, the membrane would be essentially impermeable to Ca and no energy would be necessary to maintain the Ca gradient. This condition is believed not to apply to the rat uterus since net outward movements were observed in 191 K. and Ca free K.R. and net inward Ca movements in high Ca K.R. and during metabolic inhibition. Under these modified conditions the influx of one Ca ion was thus not coupled to the efflux of another Ca ion. In order to explain an electrochemical Ca gradient across a membrane which is permeable to Ca it was necessary to postulate an active membrane transport system for Ca which derives its energy supply from cell metabolism. This postulate was confirmed by the downhill Ca movement into the cells upon metabolic inhibition by iodoacetic acid and 2,4-dinitrophenol. The present data do not rule out the

possibility that iodoacetate and dinitrophenol may have altered membrane structure so that an obligatory coupling of inward and outward movement was eliminated. This consideration arises because of the fact that higher concentrations of these metabolic inhibitors were required to cause a net uptake of calcium than to cause net downhill movements of sodium and potassium or to inhibit the ability to contract (8). Since no evidence of such an alteration in membrane structure is available and since the chemical interactions of the two inhibitors differ and since net Ca fluxes have been observed during other conditions active transport of calcium is the preferred explanation.

By studying the effects of iodoacetic acid on Ca^{45} fluxes both inward and outward it was possible to identify the active Ca transport as a Ca exclusion mechanism. This conclusion followed the observation that metabolic inhibitors increased the Ca^{45} influx, but did not affect its efflux. The finding of a low Q_{10} of 1.35 for efflux indicates that Ca efflux is likely to be a physical process, which is also in agreement with active Ca exclusion but not with extrusion. If active Ca extrusion existed in the rat uterus the efflux would necessarily have a component depending on chemical reactions so that a higher Q_{10} value would be anticipated.

An ion exclusion mechanism was first postulated by Shanes (71) in order to account for active sodium transport in unstimulated sciatic nerves of the toad. In this tissue displacement of oxygen with helium decreases K influx, increases K outflux, does not change Na outflux and increases Na influx. In cephalopod nerves metabolic inhibition only decreases K influx and Na outflux and has little

effect on K outflux and Na influx (71).

A complicating factor in washed amphibian fibres is that depolarization occurs due to an increase in membrane permeability. In an earlier paper (72) Shanes had shown that this depolarization did account for the increase in K outflux and that the increase in sodium permeability could have increased the passive Na outflux to such an extend as to mask the loss of active outward Na transport during inhibition.

However when the depolarization and presumably the increase in permeability was inhibited by 0.1% cocaine, metabolic inhibition still did not affect the Na outflux although as expected the increase in K outflux was prevented.

Shanes interpreted these results as the presence in unstimulated toad nerve of an active exchange of external Na for external K; "in this case as soon as extracellular Na enters the membrane it is carried out again in exchange for external K." Thus work is being done to reduce the inward permeability to Na, hence "sodium exclusion".

The toad nerve is also capable of active Na extrusion since restoration of oxidative metabolism caused an increase in the Na efflux.

Evidence for metabolism dependent Ca exclusion has also been presented for the guinea pig taenia-coli (14,33). Goodford (46) however found it necessary to also postulate an active Ca extrusion in this tissue on the grounds that intracellular Ca is at a lower energy level than extracellular Ca. This study did not support the presence of active Ca extrusion in rat uterus. Active extrusion was not thought to be necessary since the flux ratio $\frac{Mi}{Mo}$ could just as readily be decreased

by active exclusion which would reduce the influx (Mi), as by active extrusion which would increase the efflux (Mo).*

The active Ca transport in the rat uterus appears to have several unusual features other than the fact that it is an exclusion mechanism. Since it is not inhibited by ouabain it does not appear to be mediated through the activity of the Mg, Na and K activated adenosinetriphosphatase which is involved in active Na and K transport as extensively reviewed by Skou (48). In this respect rat uterus also differs from liver and cardiac muscle. In these tissues cardiac glycosides do induce a Ca gain (49). In the rat uterus 10^{-3} Molar ouabain does inhibit Na and K transport (50,51), and a Mg and Na activated ATPase (52). Other dissimilarities between Na and K transport in rat uterus on the one hand and Ca transport in the same tissue on the other hand were demonstrated by treatments with iodoacetic acid and with cold.

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* Ussing (47) showed that where ions move across a membrane solely under the influence of their own kinetic energy, then, provided that the likelihood of penetration by each ion is unaffected by the presence of other ions of the same species, the ratio of fluxes in the two directions will be equal to the ratio of the activities in the two solutions if allowance is made for the membrane potential:: $\frac{Mi}{Mo} = \frac{\alpha o\ Co}{\alpha i\ Ci}\ e^{ZEF/RT}$ Mi and Mo are inward and outward ion fluxes respectively. αo and αi are the activity coefficients of the ions outside and inside the cells. Co and Ci are the extra- and intracellular ion concentrations. The other symbols have their usual meaning. In the case of Ca transport across uterine smooth muscle cells active transport (as well as exchange diffusion) would decrease this flux ratio.

One tenth mMolar IAA was sufficient to cause downhill movements of Na and K after a 60 minute delay and one mMolar IAA (as used here) induced an almost immediate downhill movement of K and Na (8). In contrast the Ca gradient was not affected by 1 mM IAA until after a latent period of 70 minutes.* The absence of a net Ca gain when uteri were left at 4°C for 24 hours was unexpected and rather difficult to explain. In rat uterus, cold (below 17°C) causes downhill movements of Na and K and in guinea pig taenia-coli a net Ca gain was induced (14). The results with IAA suggest that as the energy supply starts to fail (ATP available for active cation transport decreases) Ca exclusion is affected some time after Na and K active transport are inhibited. Perhaps this could be due to a shifting of a greater proportion of metabolic energy to active Ca transport when the total energy available falls to a low level. Such a mechanism would make it possible to explain the failure of cold to induce a net Ca uptake. The depolarization which accompanies the reduction in temperature (4) would as demonstrated by the high K depolarization experiments tend to reduce the influx. The inhibition of metabolism by cold would cause a decrease in Ca exclusion and thus an increase in influx. However if the small amount of metabolic energy available is shifted to some extent from other energy requiring processes to Ca exclusion it would still be possible to maintain the Ca gradient.

Unfortunately the above hypotheses lack very much in experimental

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* The length of the delay appears to be related to the dosage of IAA. In an experiment not listed under results the latent period for the IAA induced Ca uptake was 50 minutes when IAA conc. was 2 mMolar and 200 minutes when IAA conc. was .1 mMolar.

evidence. It would be of great importance in this context to know the levels of ATP under the same experimental conditions.

Calcium binding.

Although it is generally accepted that only a small portion of the cellular Ca is ionized as has been demonstrated in intact skeletal muscle fibers (30) and squid axon (38), it is important to demonstrate that in the rat uterus intracellular Ca binding indeed occurs. Unfortunately it was not possible to measure such binding under control conditions due to the influence of membrane potential and active Ca exclusion on Ca distribution. However upon prolonged metabolic inhibition cellular Ca binding was clearly demonstrated. The results indicated that Ca was probably bound to proteins.

The next important question is: how far could this type of binding, demonstrated after metabolic inhibition, lower the free cytoplasmic Ca concentration? An approximate calculation using an intracellular exchangeable Ca concentration of 3 mMoles/liter cell water, an apparent binding constant of 110 $\frac{1}{M}$, and a total concentration of cellular binding sites of 45 mMoles/liter of cell water showed the expected free cytoplasmic Ca concentration to be 0.55 mMoles/liter cell water. Hasselbach in a recent review (41) discusses the very compelling evidence for an intracellular ionic Ca concentration in relaxed skeletal muscle not exceeding 10^{-7} Molar. Filo, Bohr and Ruegg (53) showed that glycerenated smooth muscle has the same threshold concentration of Ca for contraction (between 10^{-6}M - 10^{-7}M) as glycerenated skeletal muscle. It is thus very probable that the ionic Ca concentration in the cytoplasm of relaxed uterine smooth muscle is in the order

of 10^{-7} Molar, or 5500 times lower than the expected concentration if only passive Ca binding was responsible for lowering the cytoplasmic ionic Ca concentration. In this study no account was taken of Ca binding by high energy phosphates such as adenosine-triphosphate and creatine phosphate since these compounds were undoubtedly depleted after five hours treatment with 1 mMolar iodoacetic acid. However Nanninga's calculations (54) show that even in the presence of these compounds the ionic cytoplasmic Ca concentration would be three or four orders greater than that compatible with relaxed actomyosin.

What then is responsible for the reduction of ionic cytoplasmic calcium to a concentration below 10^{-7} Molar? In skeletal and cardiac muscle the answer to this question is almost an established fact (see review by Hasselbach 41), namely, that the endoplasmic reticulum actively accumulates Ca from the cytoplasm. The above evidence and calculations show that such an explanation is also necessary for a low cytoplasmic free Ca concentration in rat uterine smooth muscle. The endoplasmic reticulum is not very well developed in smooth muscle, but since the rate of relaxation, which depends on the rate of removal of Ca (41, 55), is much slower than in skeletal muscle this may be expected. All smooth muscles investigated exhibit a number of small vesicles close to the cell membranes and what look like pinocytotic in pocketings of the membrane, and tubule like membrane structures in the cytoplasm which are usually quite scarce (20, 56-59). However in some smooth muscle (vas deferens of rat) the tubular part of the endoplasmic reticulum is extensive (57). That the endoplasmic reticulum may indeed play a role in active ion transport is suggested by the fact that it has ATPase activity, especially the vesicles close to the membrane and the

inpocketings, as demonstrated by cytochemical staining techniques (58,59). In conclusion the experiments on Ca binding and other evidence cited strongly suggest that intracellular active Ca accumulation by certain cellular structures removes Ca from the cytoplasm of rat uterine smooth muscle. The structures which use metabolic energy to accumulate Ca are very probably the endoplasmic reticulum and perhaps the inner part of the cell membrane.

The role of Ca in excitation and contraction of rat uterine smooth muscle.

In a short but comprehensive review Schatzmann (43) discusses the evidence for the presence in smooth muscle of a Na carrier system regulated by Ca.

This theory was developed by Hodgkin and Huxley (60), Weidmann (61) and Frankenhäuser and Hodgkin (62) and proposes that the system carrying Na during the action potential can be present; (1) at rest in a readily available state, (2) in an active form and (3) in an inactive or refractory state. Ca seems to increase the available resting form on account of the refractory form and on the other hand to promote the inactivation after the system has been thrown into action. It is further assumed that the resting form of the Na transfer system is a Ca complex which upon depolarization dissociates making available sites for Na previously occupied by Ca.

In the light of the above theory it seemed very exciting that high potassium depolarization released Ca^{45} from a Ca fraction which had properties which suggested its site to be in the cell membranes. However acetylcholine which initiates action potentials and thus is also expected to cause dissociation of the Ca complex to convert it into

a Na carrying system did not appear to release Ca^{45} from the same fraction, which can at present thus not be identified with Ca regulating membrane excitability.

Schatzmann (35,43) also found a difference between the actions of acetylcholine and high K on Ca^{45} release. Both agents increased the rate of loss but the effect of acetylcholine was sustained whereas high K caused a transient increase in efflux. In the studies reported here no obvious effects of acetylcholine on efflux could be demonstrated. The observation reported here that acetylcholine did not release Ca from the Ca fraction affected by K depolarization is in agreement with the postulate that drug stimulation releases Ca from the sequestered sites while K depolarization releases Ca from superficial sites (17).

The main evidence for the essential role of Ca in contraction of smooth muscle is that depletion of smooth muscle Ca reversibly prevents all contractile responses (see review by Daniel 19). The mechanism by which Ca links excitation to contraction is incompletely understood, and likely consists of several separate actions. The final step, the details of which are still unknown, is probably the interaction of Ca with the actomyosin complex and ATP resulting in the sliding of the actin filaments past the myosin filaments leading to shortening of the sarcomeres (55,41,63,64). The influence of high potassium and acetylcholine induced contractions on Ca movements were studied to elucidate the source of the Ca which raises the cytoplasmic Ca concentration above the threshold for contraction. These two procedures would be expected to reflect the in vivo events, since acetylcholine is a physiological stimulant and high K depolarization is thought to initiate the same train of events as depolarization during an action potential (65-67).

The two views currently held for smooth muscle are that

excitation increases membrane permeability causing an increased Ca influx with the subsequent rise in free cytoplasmic Ca initiating contraction (13, 14, 16, 68) or that excitation liberates some bound Ca from the membrane into the cytoplasm and at the same time the release of Ca from the membrane destabilizes it leading to an increased Ca influx (5, 19). If the source of the raised cytoplasmic Ca during contraction is indeed entirely or partly extracellular Ca, then such procedures as acetylcholine and high K which lead to contraction should initiate an increased Ca^{45} influx from a labelled bathing medium and/or a net Ca uptake. Instead these studies in rat uterus showed that acetylcholine has no such effect or possibly slightly reduced Ca^{45} influx and that high potassium depolarization caused a decreased Ca^{45} influx and a net Ca efflux. These results are not compatible with the increased inward Ca permeability theory and indicate that excitation in rat uterine smooth muscle leads to release of Ca from cellular sites such as membrane bound Ca and/or Ca stored in the endoplasmic reticulum. This conclusion is also supported by the evidence, discussed previously, for a cellular site possibly the endoplasmic reticulum which actively accumulates Ca from the cytoplasm. Thus it seems probable that under physiological conditions in rat uterine smooth muscle excitation initiates the release of Ca from the endoplasmic reticulum and possibly the cell membrane leading to contraction. Relaxation then follows the removal of Ca from the cytoplasm by these same structures. In other words the rat uterus would have essentially the same mechanism initiating contraction and relaxation as skeletal muscle (41, 63, 69).

This hypothesis for rat uterine smooth muscle will undoubtedly not explain all the results obtained by experimentation on the contraction

of smooth muscle for the following main reasons. There are likely to be more Ca dependent steps involved in excitation contraction coupling besides the final elevation of cytoplasmic free Ca. This probability was indicated by Frank's (65) results which showed that some divalent ions which did not affect the contractile proteins themselves could substitute for Ca in supporting contractures induced by high K depolarization. Secondly Bianchi (66) demonstrated three possible ways by which the cytoplasmic free Ca could be raised by agents inducing contraction; (1) increased Ca permeability and influx, (2) release of Ca from intracellular binding sites, (3) interference with rebinding preventing relaxation. In this thesis these possible ways are not excluded but it is hypothesized that for rat uterus the physiological process is the release of bound Ca. Finally variation between tissues and species is the rule, so that the above postulated mechanism may not apply to all smooth muscle. The few parallel studies on Ca^{45} and net Ca uptake in smooth muscle will now be considered.

Schatzmann (35,43) was unable to demonstrate an increase in Ca^{45} uptake upon high K depolarization or acetylcholine exposure of the guinea pig taenia-coli. Urakawa and Holland (15) found a small increase in total Ca concentration and a marked increase in Ca^{45} influx associated with a sustained contracture induced by 40 mMolar KCl. An increase in Ca^{45} influx but not in total Ca was associated with a transient high K induced contracture in glucose free medium. However an uncertainty is introduced by the fact that the tissues were washed for 4 minutes before analysing for Ca^{45} in the influx experiments. Since the results of Bauer et al. (14) showed guinea pig taenia-coli to be almost 90% exchanged during the first 4 minutes of influx, the results

of Urakawa and Holland could also be interpreted as a slower Ca^{45} efflux following Ca^{45} loading during a contracture. With regards the small net Ca uptake the possibility was not excluded that this could have resulted from an expulsion of extracellular fluid which had a lower Ca concentration (1.8 mMolar) than the tissue (2.7 mM/kg wet wt.). Such an effect would also explain why no increase in tissue Ca was found after the twitch like contraction, since the muscle was again relaxed before analysis (see table 3 and Fig. 1 B of ref. 15). Sperelakis (36) did demonstrate an increased Ca^{45} uptake during high K depolarization of the smooth muscle of cat intestine. In this tissue the cellular Ca exchanges much more slowly (t 1/2 = 60 min.) than taenia-coli of the guinea pig. Bauer et al. did find a correlation between Ca uptake and tension development, but did not use high K or acetylcholine to induce contractions.

In depolarized smooth muscle of taenia-coli (13) and of rat uterus (this thesis) acetylcholine did increase the Ca influx over the depolarized controls, but this uptake was still less than the uptake by the polarized untreated controls in the case of rat uterus.

Ca transport through the cell membrane.

In order to explain the Ca exchange across the rat uterine cell membrane, the membrane will be considered similar to the phospholipid-cholesterol membrane studied by Mikulecky and Tobias (27). Their artificial membrane has an aqueous phase with fixed negative charges as originally proposed by Teorell (70), but differs from Teorell's theoretical model in that Ca complexes with the fixed negative sites. Using this model for smooth muscle membranes it is proposed that Ca leaving the cells

first combines with sites on the inside surface of the membrane. It then dissociates into the aqueous phase of the cell membrane and complexes with the next negative site. This process repeats itself in a random manner until the Ca ion in question is bound to the outer surface of the sarcolemma from which it then dissociates into the extracellular space.

Removal of Ca from the extracellular space could then slow the Ca^{45} efflux in two ways. (1) The release of Ca from the outer surface could be retarded. (2) Desaturation of the sites in the membrane might slow the passage of Ca through the membrane. In the latter case the slowing might be due to a large number of complexing steps in membrane transit due to desaturation of the sites. It is interesting that in the artificial phospholipid cholesterol membrane of Mikulecky and Tobias (27) a low Ca concentration retarded the equilibration of membrane Ca with external Ca. In this case a greater number of vacant binding sites may have slowed the Ca penetration into the phospholipid cholesterol complex of the millipore filter.

EDTA on the other hand may accelerate the dissociation of Ca from the outer surface. It is more difficult to see how EDTA would increase the passage of Ca through the bulk of the membrane phase unless it could penetrate some distance into the aqueous phase of the membrane. If this were possible EDTA might conceivably prevent the complexing of Ca with the anionic membrane sites by Ca chelation. Whatever the mechanism for the reduced efflux into Ca free solution the sites involved show some specificity in apparently being able to differentiate between Ca and Mg. Sr could substitute completely for Ca but Ba substituted only to a limited extent. The fact that Na did not affect the Ca efflux

into Ca free medium probably means that Na does not compete for the Ca transport sites in rat uterus, which was also indicated by the failure of Na concentration to affect the total tissue Ca concentration.

According to the two Ca fraction theory as elaborated by Daniel (17) the superficial fraction is located at the cell surface. This fraction maintains membrane excitability, supports depolarization induced contractions, and looses its Ca at a rapid rate when exposed to a Ca free medium.

The second Ca fraction is called the sequestered Ca; it supports drug induced contractions, looses its Ca at a slow rate into Ca free medium and is believed to be located below the cell surface.

Daniel (5) and Marshall (73) have shown that Sr can replace Ca in maintaining membrane potential and spontaneous action potentials. However when Ba was substituted for Ca the cells were inexcitable. Thus it seemed that Sr but not Ba has affinity for the superficial Ca binding sites.

Both Sr and Ba could substitute for Ca in supporting acetylcholine induced contractions. However the affinities of Sr and Ba for the sequestering sites seemed much lower than the Ca affinity since the Sr and Ba supported contractile effects were washed out much more rapidly than the Ca supported contractions.

Thus it appears that Sr is more like Ca in its action at the superficial Ca binding sites and more like Ba with respect to the lack of affinity for sequestering sites.

Since it was postulated that the ability of Ca, Sr, and Ba to increase the Ca^{45} efflux from rat uteri indicated affinity for Ca binding sites the results (see Fig. 29,33) indicate that the Ca and Sr affinities for

these sites are much closer than are the Sr and Ba affinities. The parallelism between the relative affinities of Ca, Sr and Ba for the superficial sites on the one hand and for the sites involved in Ca^{45} efflux on the other thus supports the postulate that membrane binding sites are involved in Ca transport and that these sites may function in the regulation of membrane excitability.

The above hypothesis of the Ca exchange through the membrane is also able to explain qualitatively the results obtained with depolarization. While changing between sites the Ca is in ionic form and is acted upon by the voltage gradient. In polarized cells this gradient promotes the passage of Ca through the membrane in an inward direction. Upon depolarization the inward electrical driving force is diminished leading to the observed reduction in Ca^{45} influx. The reduction in membrane potential should increase the efflux of cellular Ca at the same time it reduces the influx. Such an effect was not observed. In the discussion on contraction it was postulated that depolarization led to a release of Ca from the inside of the membrane. Such a decrease in affinity might tend to reduce the efflux of Ca^{45} by interfering with its first step, attachment to the inside of the membrane, and might obscure the acceleration of the passage of Ca through the membrane. Although this last explanation is quite speculative it serves to show that the results do not disagree with the hypothesis postulated earlier in the discussion.

CONCLUSIONS.

The evidence presented in this thesis is consistent with the following model for Ca exchange and distribution in the rat uterine smooth muscle.

Of the total 2.25mMole Ca/kg wet wt., 0.69mM is ionic Ca in the extracellular space, 0.11 mM is reversibly bound in the extracellular space, 1.0 mM is exchangeable intracellular Ca, and 0.45 mM is inexchangeable and may be in the form of tightly bound Ca in both the intra- and extracellular space.

The ionic Ca in the cytoplasm is extremely low (less than 10^{-7} M at rest and approximately 10^{-5} during maximal activation). More than 99% of the exchangeable cellular Ca is bound to molecules of the intracellular organelles and some is stored within the limiting membranes of the endoplasmic reticulum and mitochondria. Although passive binding was demonstrated it was not sufficient to lower the cytoplasmic Ca concentration to a level compatible with relaxation. Thus metabolic energy is used by the endoplasmic reticulum and perhaps the inner surface of the cell membrane to remove Ca from the cytoplasm.

Upon excitation by membrane depolarization Ca is released from these structures into the cytoplasm where it reacts with the actomyosin and ATP to cause contraction. Relaxation follows the active removal of Ca from the cytoplasm by these same structures. The fraction of the cellular Ca regulating contraction and relaxation need only be a small part of the exchangeable cellular Ca (10% or less)*.

The exchange of cellular Ca is influenced by the gradation of strength of cellular Ca binding and by Ca transport through the membrane. The membrane Ca transport occurs in three phases; binding of Ca to one side of the membrane, passage through the membrane involving a series of complex formations with anionic fixed sites, and the release of Ca on the other side of the membrane. The passive transport of Ca through the membrane appears to be a physical process probably involving diffusion and the formation and breaking of ionic bonds. The Ca transport sites appear specific for Ca and Sr, less so for Ba and non-specific for Mg and Na.

The electrochemical Ca gradient (internal Ca concentration isfar below its equilibrium value) is maintained by an active Ca exclusion mechanism probably located near the outer surface of the membrane.

Evidence was reported for a membrane Ca fraction which released Ca when the extracellular K concentration was increased. This fraction appeared to be different from the Ca fraction which regulates membrane excitability. No information was obtained about this latter Ca fraction nor on the actual mechanisms of Ca exclusion and the mechanism whereby depolarization causes the release of Ca from intracellular storage sites.

* Hasselbach (41) states that if maximal activity of actomyosin demands the binding of 1 mole of Ca to every mole of L-myosin and zero activity is maintained only in complete absence of bound Ca, the amount of Ca ions to be removed from fully activated skeletal muscle to cause relaxation amounts to 0.1 μmole/cm^3.

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